Department of Food and Nutrition
University of Helsinki
Finland

Bioprocess-induced changes in wheat bran protein bioavailability, nutritional quality and technological functionality

Elisa Arte

DOCTORAL DISSERTATION

To be presented for public discussion with the permission
of the Faculty of Agriculture and Forestry of
the University of Helsinki, in Walter Hall, Agnes Sjöberginkatu 2,
on 15th November 2019, at 12 o'clock.

Helsinki 2019

Custos:
Professor Kati Katina
Department of Food and Nutrition
University of Helsinki

Supervisors:
Professor Kati Katina
Department of Food and Nutrition
University of Helsinki
Docent Emilia Nordlund
VTT Technical Research Centre
of Finland Ltd
Docent Rossana Coda
Department of Food and Nutrition
University of Helsinki

Pre-examiners:
Professor Maud Langton
Department of Molecular Sciences
Swedish University of Agricultural Sciences
Professor Charles Brennan
Department of Wine, Food and Molecular Biosciences
Lincoln University

Opponent:
Professor Christophe Courtin
Department of Microbial and Molecular Systems
KU Leuven

ISBN 978-951-51-5576-4 (pbk.)
ISBN 978-951-51-5577-1 (PDF)
ISSN 0355-1180

Unigrafia
Helsinki 2019

Abstract

Wheat is the world's most important staple food, providing one-fifth of the daily protein consumed globally. However, the majority of wheat is used as refined flour, in which the nutritionally superior bran layers and germ are removed during milling, thus producing yearly a massive amount of underutilised food side streams. Better exploitation of the side streams and development of new plant-based protein ingredients are required to ensure the future global demand for food protein. This study aimed to examine hydrolytic enzymes and lactic acid fermentation as tools to improve the bioavailability, nutritional quality and technological properties of wheat bran proteins for food applications.

The study showed that proteolytic activity, either by endogenous or exogenous enzymes, was crucial for increasing protein liberation and solubilisation from wheat bran, whereas microbial activity was required for improving the nutritional quality of the proteins. The application of commercial carbohydrases or proteases was able to either solubilise the bran cell walls or the proteins from the residues of endosperm in bran but was not effective in liberating proteins within aleurone cells. The endogenous enzymes of wheat bran, activated by chemical acidification, increased the protein solubilisation up to 75% with a simultaneous increase in *in vitro* protein digestibility (from 14% to 20%). However, bioprocessing by lactic acid bacteria (LAB), yeast and cell wall-degrading enzymes (Depol 761P and Viscoferm) was found as the most beneficial and microbiologically safe method to improve the solubilisation and nutritional quality of bran proteins. This bioprocessing method resulted in a protein solubilisation of 52% and significantly improved the *in vitro* protein digestibility to 39%.

In this work, the bioprocessing of wheat bran by LAB and yeast, with and without cell wall-degrading enzymes and phytase prior to the production of protein isolates, was found to influence the biochemical and technological properties of the bran proteins. The bioprocessed protein isolates had significantly higher protein content (80%), presumably due to the degradation of starch and soluble arabinoxylans during the bioprocessing. In general, the bioprocessing of bran resulted in a lower protein solubilisation of the protein isolates and had no influence on the emulsifying properties of the isolates in oil-in-water emulsions. However, bioprocessing by lactic acid fermentation together with cell wall-degrading enzymes almost doubled the foaming stability. Furthermore, wheat breads were made by substituting 20% of the total energy by proteins from the isolates. Wheat breads enriched with the lactic acid fermented bran protein isolate was found to have the most optimal technological characteristics, showing delayed staling and lower firmness during four days' storage in comparison to bread enriched with a protein isolate produced without bioprocessing.

In conclusion, by utilising lactic acid fermentation in combination with selected hydrolytic enzymes, the aleurone cell walls can be degraded and the proteins liberated for microbial modification, leading to improved protein bioavailability, nutritional quality and technological functionality. This study is the first to show the potential of using bioprocessing for the development of new wheat bran-based protein ingredient for food applications.

Acknowledgements

This study was performed at the Department of Food and Nutrition, University of Helsinki and VTT Technical Research Centre of Finland Ltd during the years 2013–2019. The work was funded by the University of Helsinki Research Foundation, the Era-Net SUSFOOD, University of Helsinki Doctoral School, Lantmännen research Foundation and The Finnish Cultural Foundation. The Finnish Food Research Foundation and University of Helsinki Doctoral School kindly provided funding for a research visit to the University of Bari Aldo Moro and scientific conferences. All financial support is greatly appreciated. Fazer, Novozymes, Biocatalysts Ltd. and AB Enzymes Oy are warmly acknowledged for providing raw material and enzymes for the studies.

I wish to express my deepest gratitude to my thesis supervisors, Professor Kati Katina, Docent Emilia Nordlund and Docent Rossana Coda, for their dedication, support and guidance throughout this project. I truly appreciate that you all trusted me in fulfilling the high expectations in growing into a researcher. I thank Kati for being always approachable and encouraging, and your contagious drive and enthusiasm in cereal science is something to behold. I thank Docent Emilia Nordlund for always having the time for my questions and giving me excellent advice and encouragement during the project. I also greatly appreciate the possibilities to come to VTT for quick analysis visits after moving to Viikki. I am thankful for Docent Rossana Coda for all the valuable advice and helpful conversations about the research.

I am grateful for the scientific guidance given by the thesis advisory board – Research Professor Kaisa Poutanen, Professor Emeritus Hannu Salovaara and Docent Tuula Sontag-Strohm. I wish to thank all co-authors who assisted me during the project – Dr Ulla Holopainen for the microscopy analysis, Associate Professor Carlo Giuseppe Rizzello and Michela Verni for the experimental work done for publication II and Dr Xin Huang for assistance in the experimental work of publication III. I was lucky to have the greatest opportunity in 2014 for a four-month research visit to the University of Bari Aldo Moro, and I am grateful for Professor Marco Gobbetti and Associate Professor Carlo Giuseppe Rizzello for enabling me this once-in-a-lifetime experience. Dr Jose Curiel and Dr Lluana Nionelli are also warmly acknowledged for teaching and guiding me during the visit. I thank my pre-examiners, Professor Maud Langton and Professor Charles Brennan, for their careful pre-examination of the thesis.

I would not be at this point without all the assistance given in lab by the skilled technical staff and colleagues at the University and VTT; my sincere thanks to Outi Brinck, Arja Viljamaa, Eero Mattila, Mikko Kangas, Miikka Olin, Taru Rautavesi, Minnamari Edelmann, Outi Nivala, Riitta Alander, Susanna Kariluoto, Juha-Pekka Järvinen and Päivi Sorvali. My peers, Iida, Hanna N., Päivi, Ida and many others, are thanked for the cheerful lunches and coffee breaks, exchange of experience and supportive and positive atmosphere at the University. A special word of thanks goes to Hanna Koivula, with whom we have had many fun moments and laughs over the last years at Viikki. I also thank you for all the advice and encouragement related

to my PhD studies.

My dear friends outside the university, I thank for all the joy you give me and for reminding me that life goes on outside of PhD studies. I warmly thank my extended family, Leena, Ilkka, Rosi, Alisa and Antti, for the support they have given in so many ways.

I am eternally grateful to my parents for their love, care and encouragement throughout my life. Without the endless help and advice from my mother, I would have not been able to carry out my PhD. Dad would be so proud of me! I thank my brothers and their spouses, Lauri and Silja, and Eero and Marjukka, for the support throughout the years.

Finally, I owe my dearest gratitude to my beloved husband, Anssi. Your love, support, positivity and never-ending patience with me is priceless and keeps me going every day. I dedicate this thesis to our son, Alvar, the best part of us.

Helsinki, October 2019

Elisa Arte

Contents

List of original publications

This thesis is based on the following publications:

I Arte E, Katina K, Holopainen-Mantila U, Nordlund E. 2016. Effect of hydrolyzing enzymes on wheat bran cell wall integrity and protein solubility. Cereal Chemistry 93: 162–171.

II Arte E, Rizzello C.G, Verni M, Nordlund E, Katina K, Coda R. 2015. Impact of enzymatic and microbial bioprocessing on protein modification and nutritional properties of wheat bran. Journal of Agricultural and Food Chemistry 63: 8685–8693.

III Arte E, Huang X, Nordlund E, Katina K. 2019. Biochemical characterization and technofunctional properties of bioprocessed wheat bran protein isolates. Food Chemistry 289: 103–111.

The publications are referred to in the text by their Roman numerals.

Author's contributions

I Elisa Arte was responsible for planning the study with her supervisors and for the execution of the experimental work. Dr. Ulla Holopainen-Mantila carried out the fluorescence microscopy of wheat bran. Elisa Arte interpreted the data and had the main responsibility for writing the publication together with all co-authors.

II Elisa Arte was responsible for planning the study with her supervisors. She performed the bioprocessing of bran, part of the chemical analyses and the electrophoresis of the study. She had the main responsibility for the interpretation of the results and the writing of the publication with her co-authors.

III Elisa Arte was responsible for planning the study with her supervisors and for the execution of the experimental work. She had the main responsibility for the data analysis, statistical analysis and interpretation of the results. She had the main responsibility for writing the publication together with her co-authors.

Abbreviations

AACC	American Association of Cereal Chemists
AOAC	Association of Official Agricultural Chemists
ATP	Adenosine triphosphate
AX	Arabinoxylan
A/X	Arabinose to xylose ratio
EAA	Essential amino acid
FAO	Food and Agriculture Organization of the United Nations
HMW	High molecular weight
HPAEC-PAD	High-performance anion exchange chromatography with pulsed amperometric detection
LAB	Lactic acid bacteria
MW	Molecular weight
pI	Isoelectric point
SDF	Soluble dietary fibre
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide
TAXI	Triticum aestivum xylanase inhibitors
WE pentosan	Water-extractable pentosan

1 Introduction

The global population will grow by 2.3 billion in the next 30 years and will need 70% more food than today (FAO 2019a). The latest report by the Intergovernmental Panel on Climate Change stated that global warming will cause more uncertainty in food production and increase global food insecurity (FAO 2019a). The production of animal-based protein is untenable at the rates produced and consumed today and should be drastically decreased to lower its negative environmental impacts (Willett et al. 2019). Nevertheless, dietary proteins are essential for life and wellbeing, thus protein and amino acids must be obtained from the diet since they cannot be synthesised from carbohydrates or fats in the body. To produce sustainable food and especially protein for the future global needs, the existing plant-based protein sources and side streams of food production need to be utilised more efficiently. Fortunately, the growing awareness of the environmental and health effects of excess meat production and consumption has increased consumer interest towards new, sustainable plant-based protein alternatives.

The change from animal-based proteins into plant-based proteins has several technological and nutritional challenges in regard to food products and diets. The reasons for the underutilisation of plant proteins have been stated to be (1) the inferior nutritional value as compared to animal protein, (2) the technological functionality due to high protein molecular weight (MW) and poor solubility in water and (3) the high economical input to recover and purify the proteins (Day 2013).

Globally, wheat is the most important staple food, providing one-fifth of the daily calories and protein consumed (Shiferaw et al. 2013). As stated by Peña-Bautista et al. (2017), 'Wheat is the most versatile grain among the cereals for the preparation of diverse foods, providing more calories and proteins to the global population than any other agricultural food'. Unfortunately, wheat is used mainly as refined flour due to the superior technological and sensory properties and better consumer acceptability of the products. Hence, the bran fraction that contains the majority of the nutritionally beneficial compounds is left out. Instead of being used as food, wheat bran is mainly used as feed and as raw material in biofuel production. Wheat bran contains up to 18% protein, making it a potential plant protein source. The challenge in utilising bran proteins as food is that they are located in the aleurone cells, surrounded by cell walls constructed of insoluble dietary fibre. Since the insoluble dietary fibre is not fully digested in the gastrointestinal tract, the proteins within the bran matrix are not liberated nor completely digested.

This study focuses on the potentiality of bioprocessing with lactic acid fermentation and hydrolytic enzymes to improve the liberation, bioavailability and technological properties of wheat bran proteins. Lactic acid fermentation is an ancient and widely used method of transforming raw material into food products, such as bread, beer and yoghurt. In fact, fermented foods cover one-third of all the food consumed (Peña-Bautista et al. 2017). Moreover, hydrolytic enzymes are widely used in the food and agroindustry to improve nutritional, technological and sensory properties and the final quality of the product.

Treatments with enzyme-aided lactic acid fermentation have been applied to improve the technological, sensory and nutritional properties of cereal brans. Studies have shown that bran bioprocessing improves the bioavailability of minerals and phenolic compounds and increases the *in vitro* digestibility of proteins as well as improves the baking properties of bran-enriched breads (Lopez et al. 2001; Leenhardt et al. 2005; Lioger et al. 2007; Anson et al. 2009; Katina et al. 2012; Coda et al. 2014a; 2014b; Hartikainen et al. 2014). Despite several studies on this subject, the impact of bioprocessing on cereal bran protein quality, bioavailability and technofunctional properties has been poorly established, especially with wheat bran.

2 Review of the literature

2.1 Wheat bran side stream

2.1.1 Production and milling of wheat

Wheat (*Triticum aestivum* L.) is the most widely cultivated and third most-produced crop in the world (FAO 2019b). According to the Food and Agriculture Organization of the United Nations' (FAO) database (2019b), the global wheat production was 740 M tons in 2017. Two-thirds of wheat is produced in the European Union (250 M tons), and Eastern and Southern Asia (133 and 138 M tons, respectively). As reviewed by Shiferaw et al. (2013), around 70% of all wheat produced is used as food, the rest being used as feed and raw material for industrial processing. Developed countries consume wheat to a larger extent, with an average of 95 kg/capita/year, whereas in developing countries the average consumption is nearly half that, 56 kg/capita/year. The greatest amounts of wheat are consumed in Central and West Asia and North Africa, where wheat products comprise 40–47% of the total daily dietary energy. In Europe, the wheat consumption is 105 kg/year/capita, providing 20% of the daily dietary intake for both calories and proteins. The wheat consumed as food is used almost entirely as refined flour, and no data are available about the global production and consumption rates of whole-grain wheat or bran. Though, according to the United States Department of Agriculture (USDA), from the total wheat flour produced in the USA, only 5% was whole wheat flour (NASS 2018).

The first step in transforming wheat into a food ingredient is milling. The aim of milling is to separate the kernel outer layers and germ from the starchy endosperm, due to their detrimental effects on storage, processing and the end-quality of products. The majority of the side streams are used as feed or as raw material in biofuel production but also to some extent in cereal-based foods to increase dietary fibre content. Several milling by-products are collected from different stages of common commercial wheat milling. The by-products are defined as coarse bran, shorts (bran with attached endosperm and some germ), middlings and millrun (fine and coarse particles of bran, shorts, germ and flour) and red dog (low-grade flour containing bran, germ and endosperm) (Delcour and Hoseney 2010). As the milling process is highly tuned for producing fine wheat flour, the side streams are collected without 'standardisation' of the final composition (Elliott et al. 2002). Depending on the milling technique and wheat cultivar, the extraction rate of wheat flour ranges between 73–77% (Elliott et al. 2002). With an annual wheat production around 740 M tons, of which 70% is used as food, it can be calculated that up to 120 M tons of milling side streams are unutilised as food every year (Shiferaw et al. 2013).

2.1.2 Bran composition and structure

The bran comprises 13–20% of the kernel weight and is composed of several layers (Barron et al. 2011). Commonly, the layers are divided into three main fractions from the periphery to the centre, including the pericarp, the testa and hyaline layer and the aleurone layer (Figure 1) (Brouns et al. 2012). The pericarp of bran comprises 24–38%, the seed coats 22–32% and the aleurone 41–64% of the bran mass (Hemery et al. 2009b; Barron et al. 2011). The variation in the reported proportions of different layers depends on the fractionation methods (hand-dissected vs. biochemical markers), wheat cultivar and environmental impacts during cultivation.

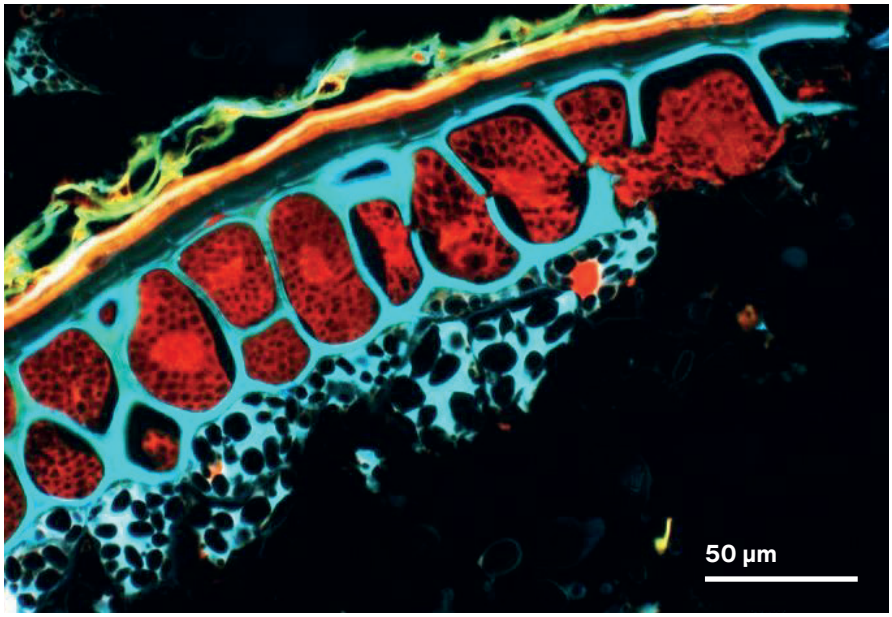

Figure 1. The microstructure of wheat bran stained by Acid Fuchsin and Calcofluor White. The cell wall structures of the pericarp layer appear light green/yellow, the testa and hyaline layer as orange and the cell walls of the aleurone layer and endosperm as light blue. The proteins are visualised as red. Image courtesy of VTT Ltd/ Ulla Holopainen-Mantila.

As mentioned, the germ is also dissected during flour milling and can be present to some extent in bran fraction obtained from milling. The germ comprises 2.5–3.5% of the grain and is rich in protein (26–35%), lipids (10–15%), minerals and bioactive compounds (Delcour and Hoseney 2010; Brandolini and Hidalgo 2012). Similarly, the starchy endosperm is not regarded as a part of the bran fraction, but remnants of endosperm are attached to the aleurone layer. Depending on the milling technique and bran particle size after sieving, the amount of starchy endosperm in the bran is generally between 9–16%, being higher in fine bran and lower in coarse bran (Antoine et al. 2004; Hemery et al. 2009a). When analysing all layers together, wheat bran contains 40–62% dietary fibre, 15–18% protein, 6–25% starch, 3–6% lipids, 2–7% phytic acid and 3–7% ash (Table 1). In addition, wheat bran is rich in bioactive compounds, such as phenolic com-

pounds, vitamins E and B and minerals (Stevenson et al. 2012). The nutrient content varies between cultivars, harvesting year, growing conditions and the hardness of the wheat (Hemery et al. 2007). Also, the proportion of starchy endosperm and germ affects the chemical composition, correlating with increased contents of starch and lipids, respectively (Chung et al. 2009; Dornez et al. 2006a).

Table 1. Relevant constituents of wheat bran and main bran fractions pericarp, testa and aleurone.*

Compound % of d.w., unless otherwise stated	Bran	Pericarp	Testa and hyaline layer	Aleurone layer
Protein	15–18	5–6	6	17–23
Ash	3–7	2	N/A	12
Lipids	3–6	0–1	N/A	5
Total dietary fibre	40–62	49–80	N/A	45–49
Arabinoxylans	22–30	45–48	38–40	18–29
A/X ratio	0.5	1.1–1.2	0.1–0.4	0.4–0.5
Cellulose	9–12	23–24	11–14	1–2
β-glucan	2–3	3–9	4–6	12–16
Lignin	2–9	1–3	3	0.5
Ferulic acid (mg/g)	4–6	3–4	5	6–7
Alkylrecorcinols (mg/100 g)	220–400	N/A	220–400	N/A
Phytic acid mg/g	2–7	N/A	N/A	7–8

* Values adapted from (Antoine et al. 2003; Barron et al. 2007; Dornez et al. 2006b; Fardet 2010; Hemery et al. 2009b, 2009a; Kamal-Eldin et al. 2009; Parker et al. 2005; Prückler et al. 2014)
N/A = Not available

The pericarp is the first defence layer of the kernel against external mechanical and pathogenic stress. It is composed of several individual layers of dead empty cells, including the outer epidermis, hypodermis, parenchyma, intermediate cells, cross cells and tube cells. These outer layers have thick cell walls composed of insoluble fibre made of cellulose and highly branched arabinoxylan (AX) with cross-links of ferulic acid and lignin (Hemery et al. 2007). The proteins in the pericarp are protective enzymes against pathogens and proteins and enzymes that strengthen the cell wall structure (Ring and Selvendran 1980; Jerkovic et al. 2010). The testa (i.e. seed coat) and hyaline layer is a hydrophobic layer completely covering the seed. The testa contains the majority of the seed alkylrecorcinols, acting as a protecting membrane against lipid peroxidation (Landberg et al. 2008; Prückler et al. 2014). The hyaline layer is composed mainly of AX, with a low arabinose to xylose ratio (A/X ratio ~ 0.1) and with cross-links of ferulic acid (Antoine et al. 2003; Barron et al. 2007; Parker et al. 2005). As in the pericarp layer, the proteins in the testa and hyaline layer are mainly enzymes (Jerkovic et al. 2010).

The wheat aleurone layer is the largest part of the bran, and it entirely covers the endosperm and germ (Barron et al. 2011). The aleurone layer acts as the nutrient storage for the seed and holds digestive endogenous

enzymes for germination (Fath et al. 2000). Almost all physiologically beneficial compounds in bran are found in the aleurone layer. For example, the aleurone layer contains 80% of the minerals in the kernel, thus having high amounts of phosphorus, potassium, magnesium, calcium, zinc and iron (Buri et al. 2004). Also, it contains significant amounts of vitamins (B and E), phenolic compounds (ferulic acid, sinapic acid and coumaric acid) and sterols found in wheat (Buri et al. 2004). The aleurone layer is constructed of living single-layer cells surrounded by dietary fibre cell walls. The cell walls are rich in AX (65%) and β-glucan (30%), the rest being cellulose, glucomannan, structural proteins and phenolic acids (Stone 2006). In comparison to the A/X ratio of the pericarp and endosperm cell walls (1.1–1.2 and 0.8–0.9, respectively), the aleurone cell walls are less substituted (A/X ratio 0.4–0.5) (Barron et al. 2007). However, the AX is highly esterified by ferulic acid, making it mostly insoluble (Antoine et al. 2003). The β-glucan found in the aleurone layer is enriched in the outer cell walls and at the endosperm-side of the layer (Regvar et al. 2011; Jääskeläinen et al. 2013). The aleurone cells contain one large oleosome-surrounded protein storage vacuole with densely packed globoids embedded in a protein matrix (Bohn et al. 2007; Bechtel et al. 2009; Regvar et al. 2011). The protein storage vacuoles contain two types of globoids, of which type I is rich in phytate and minerals, and type II is rich in niacin (Bohn et al. 2007; Bechtel et al. 2009; Regvar et al. 2011). The phytic acid-rich globoids are composed of proteins (46% w/w) and phytic acid (40% w/w) associated with several minerals (i.e. phosphorus, magnesium, potassium, iron and calcium) (Bohn et al. 2007). The majority of the wheat phytic acid is located in the aleurone (80%), but some phytic acid has been identified from the germ as well (O'Dell et al. 1972; Fardet 2010).

2.2 Wheat bran proteins

Traditionally, proteins have been classified according to their solubility into albumins (water-soluble), globulins (salt-soluble), prolamins (alcohol-soluble) and glutelins (acid/base-soluble). The endosperm in wheat contains mainly prolamins and glutelins (also named gliadin and glutenin), whereas albumins and globulins are the most abundant proteins in the bran. According to Idris et al. (2003) and De Brier (2015), 33–40% of the wheat bran proteins are albumins and globulins, 11–19% gliadins and 12–26% glutenins. The remaining part of the proteins (18%) cannot be solubilised by the Osborne solvents and is regarded as non-proteinous nitrogen and insoluble protein. According to De Brier et al. (2015), the gliadins in wheat bran mainly originates from the endosperm, thus the content depends on the amount of endosperm remaining in the bran fraction.

The high content of albumins and globulins in the bran is associated with the nutritionally superior amino acid content of bran in comparison to wheat endosperm. Bran proteins contain three times more lysine and are rich in other essential and conditionally essential amino acids, such as

histidine, threonine, valine, glycine and arginine, compared to the amino acids found in the starchy endosperm (Shewry et al. 2009; De Brier et al. 2015). Bran albumins and globulins contain significantly lower amounts of proline, glutamine/glutamic acid and phenylalanine, which are known to be prevalent amino acids in wheat gliadins and glutenins.

The proteins have a multifunctional role in the seed and can be divided according to their role in the seed as storage proteins, structural proteins and enzyme proteins. Within the bran layers, the outer and intermediate layers contain mainly stress- and defence-related enzymes and structural proteins, whereas the aleurone layer contains storage protein, metabolically active enzymes and defence and inhibitor enzymes (Jerkovic et al. 2010). Since the enzyme proteins have a significant role in this study, they are discussed separately in section 2.3.

2.2.1 Storage proteins

The storage proteins in bran are located in the protein storage vacuoles of the aleurone cells and account for 47% of the total bran proteins and up to 66% of the proteins in the aleurone layer (Kriz 1999; Jerkovic et al. 2010; Meziani et al. 2012). Their role is to provide nitrogen and carbon to the seedling during germination. The storage proteins are globulins belonging to the 7S globulin family and are similar to the aleurone proteins of other dicotyledonous plants and vicilins in legumes (Kriz 1999). The cereal 7S globulins are trimeric proteins and consist of polypeptides, with an MW of 40,000, 50–55,000 and 70,000 g/mol (Kriz 1999; Chaquilla-Quilca et al. 2018). Three types of globulins have been identified from wheat bran, globulin-1, globulin-3 and grain softness protein-1 (Laubin et al. 2008; Meziani et al. 2012; Chaquilla-Quilca et al. 2018). In contrast to legumes and oilseeds, the storage 7S globulins are a minor class of storage proteins in wheat, thus not yet thoroughly characterised.

2.2.2 Structural proteins

The wheat aleurone cell walls contain two-times the amount of structural proteins (1% w/w) in comparison to the endosperm cell walls but are similar to each other in terms of amino acid composition (Rhodes and Stone 2002). The structural proteins in the outer layers of the wheat bran have not yet been characterised in detail. Yet, a small amount of structural proteins have been reported to be within the cell walls of the outer pericarp, likely forming covalent cross-links between ferulic acid and lignin (Ring and Selvendran 1980; Stone 2006). According to Rhodes and Stone (2002), the aleurone cell walls are known to contain at least three protein types: glycine-rich (37–86 mol%), proline-rich (11–39 mol%) and serine-rich proteins (11–23 mol%). The proline-rich proteins are associated with the cell wall AX of a low substitution ratio. The authors suggest that the proteins are cross-linked to the cell wall AX and β-glucan either through ferulic acid or by protein-protein linkage.

2.3 Wheat bran enzymes

Wheat bran contains a significant pool of enzymes that can be classified by their role as stress- and defence enzymes and enzymes functioning in tissue-strengthening and kernel metabolism. The metabolic enzymes are various hydrolases acting on the kernel compounds, such as the cell wall polysaccharides, protein and starch. The endogenous enzymes can be either present in resting grain or activated during food processes such as fermentation or are synthesised during germination. In this section, the most relevant bran enzymes regarding this thesis are reviewed – the defensive enzymes and metabolic enzymes active in hydrolysing carbohydrates, proteins and phytic acid.

2.3.1 Defensive enzymes

The outer layers of bran contain various enzymes defending the inner kernel parts. According to the comprehensive study by Jerkovic et al. (2010), the proteins in the outermost layer (pericarp) are mainly defensive enzymes against fungi and bacteria (i.e. oxalate oxidase, peroxidase and polyphenol oxidase). The intermediate layer has more diversity in the pool of endogenous enzymes, containing defensive PR proteins, such as wheatwin1 and chitinase, and enzyme inhibitors, such as XIP (xylanase-inhibiting proteins) and TAXI (Triticum aestivum xylanase inhibitor). The majority of the endogenous enzymes in the aleurone layer has a role in grain metabolism during seed growth and development and during germination. In addition, the aleurone layer contains some defensive proteins such as the wheat α-amylase/subtilisin inhibitor (WASI) and class II chitinase (signalling enzyme).

2.3.2 Carbohydrate active enzymes

The most relevant cell wall-degrading enzymes are carbohydrate-active hydrolases, including xylanases, arabinofuranosidases, xylosidases and β-glucanases. These enzymes are most likely located in the aleurone cells although their exact location is not known (Dornez et al. 2006b; Vatandoust et al. 2012). The content of endogenous xylanases in wheat is rather low, but the total amount increases significantly with the presence of microbial xylanases in the outer layers of bran. According to Dornez et al. (2006a), over 90% of the xylanases in bran and almost all xylanases in the pericarp layer are of microbial origin, thus not endogenous to wheat (Dornez et al. 2006a). The pH optimum of the actual endogenous xylanase has not been reported, but a pH of around 5 has been used in studies to examine the xylanase activity in wheat brans (Cleemput et al. 1997; Dornez et al. 2006a; 2006b). The microbial xylanases are known to be most active in a pH range of 5–7 and the fungal xylanases in the range of 3–10 (Courtin and Delcour 2002). The arabinofuranosidases and xylosidases act synergistically with the xylanases in the hydrolysis of cell wall AX. Whereas the (1,3–1,4)-β-glucanase in wheat is active against the cell wall β-glucan, the (1,3)-β-glucanase belong to the pathogenesis-related proteins, having a defensive role in the grain (Jerkovic et al. 2010). The activity of these carbohydrases is low in resting grains but increases upon germination (Brijs et al. 2009).

Both amylases are present in developing grain, but only β-amylase is

present in mature grain (outlined sprouting). The major role of β-amylase is starch degradation during germination. In mature grain, half of the enzyme is regarded as storage protein and bound to other proteins (serpins and glutenins) (Brijs et al. 2009). In addition to wheat bran, β-amylase has been identified in the germ and endosperm as well and has a pH activity range from 5.0 to 6.7 (Mathewson and Seabourn 1983; Skylas et al. 2000; Chaquilla-Quilca et al. 2018). The activity of α-amylase is used as a quality indicator for cereal flours. As α-amylase activates during germination, it is used to evaluate the rate of the pre-harvest sprouting of cereals. The activity of α-amylase has been reported to be higher in bran and germ than in refined wheat flour (Dornez et al. 2006b; Every et al. 2002).

2.3.3 Proteolytic enzymes

The proteolytic enzymes in cereal grains have varying activities during grain development and germination. To clarify the nomenclature of proteolytic enzymes in this thesis, the term protease is used for all proteolytic enzymes, the term proteinase to refer endoproteases and peptidase to refer exoproteases. Proteases are commonly classified according to their catalytic mechanism into aspartic proteases, serine proteases, metalloproteases and cysteine proteases.

Aspartic proteinases and serine carboxypeptidases are the main proteolytic enzymes active in resting grains (Gänzle et al. 2008). In general, the aspartic proteinases are active in a pH range of 2.0–4.5 and the serine carboxypeptidases in a pH range of 4.0–6.0 (Mikola 1986; Bleukx et al. 1998; Bleukx and Delcour 2000; Loponen et al. 2004). Galleshi and Felicioli (1994) characterised an aspartic proteinase from wheat bran with a pH optimum at 3.3. The proteinase had an MW of 66,500 g/mol and was active on wheat aleurone globulins. Aspartic proteinases have also been identified from wheat flour and commercial wheat gluten (Bleukx et al. 1998; Bleukx and Delcour 2000). These proteinases hydrolysed glutenin but had no or very low activity against wheat albumins and globulins. Serine carboxypeptidase characterised from wheat bran and grains has shown to have peptidase activities in acidic conditions, releasing aromatic amino acid residues at the C-terminus of polypeptides (Breddam et al. 1987; Dunaevsky et al. 1989). The peptidases had a weak activity on wheat gliadin when acting alone but increasing hydrolysis when acting synergistically with aspartic proteinase (Dunaevsky et al. 1989).

2.3.4 Phytase

Phytases in wheat are known to be concentrated in the bran (Okot-Kotber et al. 2003; Bohn et al. 2007). Wheat phytases belong to phosphatases that hydrolyse phytic acid, liberating the bound phosphorus and other chelated minerals during grain development and germination. According to Okot-Kotber et al. (2003), the activity of phytase is influenced by growing locality and cultivar, being higher in red wheat (2–5 FTU/g bran) than in white wheat (2–3 FTU/g bran). The activity levels of phytase is significantly enhanced (up to 5-fold) by extraction with β-glucanase and endo-xylanase. Two phytases have been identified from wheat bran. Phy1 had an optimal activity at pH 5.6–6.0 and 45 °C (Lim and Tate 1973; Nakano et al. 1999; Bohn et al. 2007). The second phytase, Phy2, has been showed to have

optimum activity at pH 7.2 (Lim and Tate 1973) and at pH 5.5 at 50 °C (Nakano et al. 1999). According to Nakano et al. (1999), both of the identified phytases were active against phytic acid. However, Phy1 also had high substrate specificity for adenosine triphosphate (ATP), whereas Phy2 had higher affinity towards p-nitrophenylphosphate and ATP than towards phytic acid.

2.4 Challenges in utilising wheat bran proteins

Cereal bran is a potential plant protein source but cannot be efficiently utilised as such. The first limiting factor is the location of storage proteins within the aleurone cells. In general, the insoluble and impermeable cell wall structure surrounding the proteins hinders the extractability and bioavailability of wheat bran proteins. Secondly, other bioactive compounds present in bran, such as phytic acid and dietary fibre, can form complexes with the protein, further limiting its bioavailability. Also, the uncontrolled activation of contaminant microorganisms or endogenous enzymes in bran can have detrimental effects during food processing.

2.4.1 Dietary fibre and digestibility of proteins

Despite the well-known positive effects of dietary fibre on human health (Slavin 2004), the insoluble dietary fibre in wheat bran hinders the bioavailability of proteins. First, the location of bran proteins inside aleurone cells surrounded by polysaccharide-constructed cell walls physically prevents the liberation of proteins within the cells and the gastric enzymes to act on the protein. Second, the higher content of highly branched AX, cellulose and lignin present in the whole bran and especially in the pericarp-rich fraction increases the bulking material in the gastrointestinal tract hindering the adsorption of bran proteins (Amrein et al. 2003; Harris et al. 2005; Brouns et al. 2012).

Coda et al. (2014a) reported that the *in vitro* digestibility of wheat bran proteins was linked to the particle size of bran. The highest protein digestibility (38%) was obtained in bran with a particle size of 400 μm and the lowest digestibility, 28%, in bran with the smallest particle size (50 μm). Amrein et al. (2003) showed that the aleurone-rich fraction from wheat bran had significantly a higher overall *in vitro* digestibility, of 28–32% (carbohydrates, fat and protein), than in whole bran (13%). Similarly, the protein *in vitro* digestibility was higher in the aleurone-rich fraction (50–57%) in comparison with the whole bran (37%). The higher digestibility of the wheat aleurone rich-fraction was also noted in a rat study by Harris et al. (2005). A large number of intestinal bacteria was adhered to the aleurone-rich fraction, leading to a formation of holes in the cell wall. The pericarp-rich fraction, on the other hand, had few bacteria adhered and still-intact cell walls. The difference in the digestibility of pericarp-rich and aleurone-rich fractions was explained by the differences in their cell wall polysaccharide compositions.

2.4.2 Phytic acid

The antinutrient effect of phytate is primarily associated with the capability to bind minerals in acidic conditions. Positively charged minerals, especially zinc, calcium, iron and magnesium, are susceptible to forming indigestible complexes with the negatively charged phosphate groups of phytic acid (Lopez et al. 2002; Coulibaly et al. 2011). However, phytic acid can form insoluble complexes with proteins as well, decreasing their solubility and bioavailability. Below the protein isoelectric pH (e.g. stomach conditions of humans), the terminal amino groups lysyl, histidyl and arginyl can be positively charged and form insoluble complexes with negatively charged phytate (Coulibaly et al. 2011). At a pH above the protein isoelectric point (pI), both proteins and phytic acid have a negative net charge but are able to form complexes through multivalent cations (Cheryan 1980). The phytic acid-protein complexes have also been shown to inhibit the activity of digestive enzymes, namely pepsin, trypsin and amylase (Cheryan 1980; Knuckles et al. 1985; Li et al. 1993; Bye et al. 2013). Since phytase is lacking in the digestive systems of humans and monogastric animals, phytic acid is not degraded during digestion, thus decreasing the bioavailability of the bound minerals and protein (Cheryan 1980).

Positive health effects of phytic acid have also been proposed. Phytic acid is known to have a role as a natural antioxidant by binding iron and preventing its participation in the oxidative Fenton reaction. In the Fenton reaction, iron is oxidised by reacting with hydrogen peroxide (or other peroxides), which results in the formation of highly oxidative and damaging free radicals (Bohn et al. 2008). The antioxidative ability of phytic acid has been suggested to be a possible inhibitor for illnesses linked to free radicals, such as colon cancer and kidney stones (Graf and Eaton 1990; Selvam 2002).

Despite the negative effect of phytic acid on proteins and minerals, a diet containing whole-grain foods (including the phytic acid) has significantly more positive than negative effects on overall human health (lowering the risk of obesity, diabetes, cardiovascular disease, etc.). The chelating effect of phytic acid in grains is not high enough compared to the high mineral and protein content, thus not overcoming the positive effects of whole grain. For example, rats fed with a whole-wheat diet had better animal growth than rats fed with refined wheat flour (Levrat-Verny et al. 1999). However, regarding wheat bran proteins, the formation of phytic acid-protein complexes should be taken into consideration during food processing and end-product quality.

2.4.3 Activation of endogenous enzymes

The pool of endogenous enzymes in cereal brans can have a positive or negative impact on food processing and on the end-quality of products. For example, the excess activation of endogenous enzymes has a detrimental effect on baking properties, but the entire malting and brewing process is based on the activation of endogenous enzymes. As reviewed by Hemdane et al. (2016), high α-amylase activity can cause excess starch degradation during baking, resulting in sticky and unmanageable dough. Also, the excessive hydrolysis of AX by xylanases can decrease water-holding capacity, subsequently resulting in sticky dough and destabilises the dough foam formation. Lipases can form off-flavours during storage by releasing fatty acids

from cereal brans (Brjis et al., 2009). Polyphenol oxidases identified from the outer layer of bran has been related to the darkening of wheat-based products (Soysal and Söylemez 2004).

Also, the activation of proteases during bran processing can influence the flavour formation and interaction with other compounds. Small molecular-sized peptides formed by proteolytic enzymes can cause a bitter taste in whole-grain products (Heiniö et al. 2012). In addition, the activated endogenous proteases can hydrolyse other compounds present in food during processing, such as degrading the gluten in bread-making, resulting in a weaker gluten network (Hemdane et al. 2016).

2.4.4 Microbial contaminants

In addition to purifying starchy endosperm, one aim of milling is to remove impurities from the flour, resulting in the redistribution and concentration of microbial contaminants to the milling by-products, bran and germ. As reviewed by Sabillon and Bianchini (2016), the microbiota of wheat grains is located mainly in the outer layer of the kernel (outer pericarp), composed of a diversity of bacteria, yeasts and moulds. Generally, the growth of microorganisms in brans can be prevented by sustaining low water activity. However, low water activity does not remove the microbes from the material, and they can remain in a dormant state (e.g. in the form of spores) in bran. In addition, the possible presence of mycotoxins can cause health risks in the end products. The negative effect of microbes can be significantly reduced by peeling, pearling and dry fractionation methods that separate the outer layers of kernel from the aleurone and endosperm (Hemery et al. 2007; Katina et al. 2007). However, these methods are not in general industrial use, thus the presence of microbes in bran, including possible pathogens, should be taken into account during food processing, where high water content and long incubation times are used.

2.5 Technologies to improve cereal bran protein utilisation

Mechanical and chemical processes, as well as bioprocesses utilising micro-organisms, can be used to modify cereal bran for improved applicability to food. These technologies, such as milling, wet-extraction and fermentation, can be used for increasing the yields of bran protein separation and extraction or for modifying the bran to improve the bioavailability, nutritional quality, and technological properties of the bran proteins. In this thesis, the term 'bioprocessing' is used for different combinations of treatments applying exogenous enzymes and/or lactic acid fermentation. The term 'fermentation' refers to lactic acid fermentation.

2.5.1 Milling and reduction of bran particle size

Usually, the bran collected from the mill is coarse bran and, as the name indicates, is made of coarse particles, with an average size between 600–

2200 μm (Zhang and Moore 1999; Greffeuille et al. 2006). The addition of coarse wheat bran has detrimental effects on the technological and sensory profile in cereal-based foods when compared to foods with refined flour (Hemdane et al. 2016). Decreasing bran particle size by further milling, grinding and sieving is as a feasible method to potentially overcome the negative effects of coarse bran on food quality and to increase the bio-accessibility of nutrients. Dry fractionation methods (e.g. electrostatic separation, ultrafine grinding, air-classification and supercritical carbon dioxide) have been studied to separate specific layers within cereal brans and to enrich valuable nutrients in the fractions (Hemery et al. 2007; Hemery et al. 2009a; Noort et al. 2010; Sibakov et al. 2011). In wheat, fractionation methods can be used to separate the pericarp layer low in proteins to enrich the aleurone fraction with higher protein content (Noort et al. 2010; Rizzello et al. 2012a). However, dry fractionation methods have not yet been applied at the industrial scale.

Although the overall protein content is not affected by milling, bran particle size reduction has been shown to improve the protein liberation and extractability and change the protein composition and nutritional profile. De Brier et al. (2015) used ball-milling (10 min) to improve bran protein extractability. Reducing bran particle size from 800 μm to 175 μm increased the total protein extraction yield (by Osborne fractionation) from 60% to 77%. The milling increased the extraction yield of albumins and globulins from 33% to 42% and of glutenins from 16% to 32% but had no impact on the extraction yield of gliadins. The authors concluded that ball-milling disrupted the aleurone cell walls, thus liberating proteins within this layer. Coda et al. (2014a) showed that by reducing wheat bran particle size from 750 μm to 50 μm, the peptide and free amino acid contents of bran water-soluble extracts were increased by 52% and 23%, respectively. Decreasing the bran particle size increases the surface area and enables better interaction with the solvent, that resulted in the improved solubilisation of nutrients (Hemery et al. 2011). Coda et al. (2014a) found a particle size of 400 μm to be optimal for *in vitro* protein digestibility, in which 39% of the proteins were digested in comparison to larger (750 μm) or smaller particle-sized brans (50 μm and 160 μm), resulting in protein *in vitro* digestibility of 29–35%. Bran particle size also influenced the protein quality indexes (i.e. essential amino acid index, biological value, nutritional index, and protein efficiency ratio) that are based on essential amino acid compositions. The highest indexes were found in bran with the largest particle size (750 μm), and a reduction of particle size to 160 μm resulted in the most significant decrease of protein quality indexes, by 22–29%, except for nutritional index, which was reduced the most (by 28%) in bran with a size of 400 μm. Hemery et al. (2011) showed that wheat bran layers do not break down simultaneously during grinding, potentially altering the bioavailability of nutrients in brans with different particle sizes. For example, the intermediate layer in bran was not easily fragmented during grinding due to high plasticity and extensibility.

2.5.2 Wet extraction and isolation methods

In general, plant proteins have poor solubility in water, thus the most common method to obtain high protein yields form cereal side streams is wet

extraction by alkaline or acidic solvents. As the majority of bran proteins are albumins and globulins, they should be readily extractable with water and saline solutions. However, as discussed above, the location within bran cell walls hinders the extractability of these proteins. Generally, protein extraction is based on the protein pI and solvent pH: at the pH outside the protein pI, the protein's electrostatic repulsion and hydration of charged residues favour the solubilisation of the protein (Damodaran 2008). In addition to extraction pH, the solubility is affected by the ionic strength, temperature and compounds physically limiting solubilisation.

High extraction yields of wheat bran proteins have been obtained by extraction with highly alkaline solutions and by sequential extraction based on the Osborne fractionation into water-, saline-, ethanol- and acid/basic-soluble proteins. Roberts et al. (1985) used an extraction pH of 12 followed by three washings with water (pH 7) to obtain a protein extraction yield of 83% from ground wheat bran. In the same study, a significant amount of proteins (72%) were also extracted with tap water at ambient temperatures (16 h) followed by three washings. In another study, by Idris et al. (2003), the same protein extraction yield (83%) from ground wheat bran was achieved by sequential extraction based on the Osborne fractionation.

De Brier et al. (2015) studied several extraction methods to obtain the highest yield of wheat bran proteins. The extraction yield of coarse wheat bran proteins remained under 20% at a pH below 8 but was increased up to 37% by increasing the extraction pH to 12. Furthermore, by adding three sequential extractions and elevating the temperature to 60 °C, the yield was increased to 55%. However, the highest protein extraction yield, 77%, was obtained by reducing the bran particle size by ball milling (10 min) to 175 μm followed by sequential extraction according to the Osborne fractionation. The authors concluded that milling disrupted the aleurone cell walls, thus improving the liberation and extractability of the proteins within cells.

The alkaline conditions often used for protein extraction can form undesired complexes and compounds. For example, the formation of phytate-protein complexes is influenced by pH. At a pH higher than the protein pI, phytate-protein complexes can be formed through multivalent cations, but at a pH above 10, the complex dissociates, and the proteins can be liberated and solubilised (Cheryan 1980; Coulibaly et al. 2011). Moreover, extraction in a highly alkaline pH (above 12) can result in protein aggregation and the formation of undesired compounds, such as lysinoalanine (Mauron 1990). Also, browning can occur during alkaline extraction from the reaction of polyphenols and proteins, thus possibly reducing consumer acceptance. Methods such as diafiltering prior to canola seed protein extraction and the separation of wheat germ proteins by reverse micelles have been studied to overcome the formation of undesired compounds (Xu and Diosady 2002; Zhu et al. 2010).

As protein extraction is often conducted in highly alkaline conditions with a low raw material to solvent ratio, the extracts are not applicable to food products as such. Precipitation and isolation methods have been applied in concentrating extracted proteins and to achieve a product with an acceptable pH (4–8). Simultaneously, the other compounds, such as sugars, ash and polyphenols, are separated from the protein fraction. In contrast to protein extraction aiming at the highest solubility of proteins, protein isolation

is based on the precipitation of the proteins at the protein pI.

Most plant food proteins exhibit pI in the range of 4–5, and a precipitation pH between 4.0–5.7 has generally been used for globulin-rich plant materials (Hettiarachchy et al. 1996; Meinlschmidt et al. 2016b; Wang et al. 1999; Zhu et al. 2010). In general, cereal bran and germ protein isolates produced by alkaline extraction and subsequent protein precipitation have protein contents in the range of 67–90% and protein extraction yields of 18–34% (Hettiarachchy et al. 1996; Prosekov et al. 2018; Wang et al. 1999). Despite the relatively high purity of protein isolates, the protein yields remain low due to the wide range of proteins in the cereal materials with differing pI. The use of multiple solvents has also been studied to improve the protein yields of isolates. As reviewed by Fabian and Ju (2011), sequential extraction by water, NaCl, ethanol and NaOH solutions can increase protein yield to above 90% of the rice bran protein isolate. However, applying multiple steps in the process can reduce the overall yield from the loss of product per extraction.

2.5.3 Bioprocessing methods

Enzymatic treatments

The increasing utilisation of enzymes in the food industry is based on the economic benefits of lowering processing costs and environmental impact while simultaneously improving the product quality with a 'clean-label' approach (Porta et al. 2011). The main food-grade enzymes are various hydrolases – carbohydrases, proteases and lipases. In addition, some hydrolases, such as lysozymes and oxidoreductases (glucose oxidases), are used for food preservation (Ramos and Malcata 2011).

The most widely used commercial or experimental enzyme preparations to hydrolyse cereal polysaccharides are various xylanases, β-glucanases, cellulases, pectinases and amylases. Usually, the commercial enzyme preparations are not 'pure' having only one type of enzyme activity but having multiple side activities, for example β-glucanases, pectinases, ferulic acid esterases and/or proteases that act synergistically enhancing the breakdown of plant polysaccharides. Furthermore, the specific enzyme and origin of the enzyme (fungal/bacterial) determine the functionality of the enzyme, such as substrate specificity, optimal pH and temperature range, and hydrolysis products (Courtin and Delcour 2002). These features in different enzyme preparations can be used for the targeted hydrolysis of cereal cell wall polysaccharides and modifications of the bran structure. Modifications of cereal brans by selected carbohydrases have been widely studied to improve the technological and nutritional properties of the dietary fibre fraction. For example, the carbohydrase-treatment of cereal brans has been used to improve the handling, textural, sensory and nutritional properties of bran extrudates and bran-enriched breads (Laurikainen et al. 1998; Sanz Penella et al. 2008; Santala et al. 2013).

As AX is the main polysaccharide in cereal bran cell walls, xylanases are the most used and studied enzymes for bran cell wall degradation. Xylanases randomly break down the xylose β-1,4-bonds of AX, eventually leading to the formation of soluble AX oligosaccharides. Generally, commercial xylanases are of microbial origin, and they preferentially act on AX with

low arabinose substitution and can be inhibited by the cereal endogenous xylanase inhibitors (Benamrouche et al. 2002; Dornez et al. 2009). Benamrouche et al. (2002) showed that 50% of the wheat bran AX can be degraded by treatment with purified bacterial xylanase. The most intense degradation was observed in the aleurone layer (low AX substitution level), where 80% of the AX was hydrolysed, whereas the outer pericarp was unaffected by xylanase. Petersson et al. (2013) reported that xylanase from *B. subtilis* with β-glucanase and ferulic acid esterase side activities was the most effective in degrading wheat aleurone cell walls. Hence, the degradation of bran cell walls by enzymatic treatments has been applied to liberate bioactive compounds and proteins within the bran layers (Waszczynskyj et al. 1981; Faulds et al. 2003; Heiniö et al. 2012; De Brier et al. 2015). For example, the combined effect of xylanases and ferulic acid esterases has been used to liberate the phenolic compounds, such as ferulic acid, bound to bran AX (Faulds et al. 2003).

Enzyme treatments of cereal brans can be used to improve protein extraction as well, resulting in higher protein yields with lower environmental impact compared to the traditional wet alkaline or acidic extraction methods (Sari et al. 2015). Table 2 presents studies on the effect of commercial or experimental enzymes on cereal AX solubilisation and protein extraction yield. In general, carbohydrase treatments of cereal brans in relatively mild conditions result in a protein extraction yield of 38.5–58%. Overall, only a few studies have been performed on the effect of enzymatic treatment on the extraction yields of wheat bran proteins. Waszczynskyj et al. (1981) treated wheat bran with cellulase, hemicellulase and pectinase and increased the protein yield from 30% to 38.5%. In the study by De Brier et al. (2015), cellulase and xylanase treatment increased the protein extraction yield from 22% to 33% and 47%, respectively, and further to 68% by applying extraction by 1% SDS prior to the enzymatic treatment. Reisinger et al. (2013) used hydrothermal pre-treatment followed by enzymatic treatment to increase wheat bran protein extraction yield to 73%. However, the high temperature used in the pre-treatment caused losses of free amino acids (lysine and arginine) to Maillard reactions and caused the formation of undesired compounds, such as toxic furfural. Jodayree et al. (2012) used commercial carbohydrase preparations – Viscozyme L, α-amylase, amyloglucosidase and Celluclast – to improve the protein content of oat bran protein isolates. The results showed that an amyloglucosidase pre-treatment for 3.5 h at 45 C resulted in the highest increase of protein content, from 54% to 82%, in the oat bran protein isolate. α-amylase and Viscozyme L pre-treatments also significantly increased the protein content of the isolates, up to 70–72%, whereas the Celluclast pre-treatment resulted in a protein content similar to the control isolate without enzymatic pre-treatment (52%). Treatments with cell wall-degrading enzymes in combination with other hydrolytic enzymes have been studied to enhance protein extraction by reducing the ability of proteins to interact with other compounds, such as phytic acid. Wang et al. (1999) showed that the combined action of xylanase and phytase was more effective in increasing the protein content (from 75% to 92%) and yield (from 34% to 75%) of rice bran isolates than the action of the enzymes individually (protein content 80–82% and protein yield 55–57%). According to Hanmoungjai et al. (2002), carbohydrase

Table 2. Examples of treatment parameters used in enzyme-aided cereal bran protein extractions, the obtained solubilisation of AX (%) and protein extraction yields (%).

Enzymatic activity	Commercial name of enzyme preparation	Dosage	Raw material	Bran to solvent ratio	t (h)	T (C)	pH	Solubilisation of AX (%)	Protein extraction yield (%)	Reference
Cellulase+ hemicellulase+ pectinase	Cellulase, hemicellulase, pectinase	222 mg 588 mg 409 mg	Wheat bran	1:5 w/v	7	37	8.5	Not analysed.	39	Waszczynskyj et al. 1981
Xylanase	Purified thermos-stable xylanase	10 U/ml	Starch -depleted wheat bran	30 g/l	24	60	N/A	50% bran AX	N/A	Benamrouche et al. 2002
Xylanase cellulase	1) Xylanase 2) Cellulase	10 000U	Wheat bran	1:12.5 w/v	4	40	5	Not analysed.	47 33	De Brier et al. 2015
Cellulase+ β-glucanase+, xylanase+ α-amylase+ protease	Cellulase + β-glucanase + xylanase + Termamyl + AMG	0.2g/ml 0.165 mg/l 0.052 g/ml 10 µl/ml 10 µl/ml	Hydrothermally pre-treated wheat bran	1:9 w/v	72	40	3.9–6.3	70% of AX	73	Reisinger et al. 2013
β-glucanase	Viscozyme L	30 FBG	Defatted oat bran	1:10 w/v	2.8	35–55	4.6	Not analysed	56	Guan and Yao 2008
α-amylase, β-glucanase, cellulase	Amylase, Viscozyme L Celluclast	87 637 U 160 FBG 700 EGU	Heat-stabilised rice bran	1:17 w/v 1:10 w/v 1:10 w/v	4.5 3.5 3.5	51 45 55	6.3 4.1 5.5	Not analysed	58 29 12	Tang et al. 2003
β-glucanase+ cellulase	1) Viscozyme L 2) Viscozyme L + Celluclast 1.5L	120 FBG 120FBG + 360 NC	Extruded rice bran flour	1:4 w/v		50 40	3.8	Not analysed	58 51	Ansharullah et al. 1997
Protease	Alcalase 2.4L Flavourzyme	0.5% of flour weight	Rice bran	1:50 w/v	Until 10% hydrolysis degree	50	8		811 88	Hamada 2000

treatment with cellulase, hemicellulase, pectinase or β-glucanase followed by peptidase treatment significantly improved the protein extraction yield of rice bran, from under 20% up to 50%. Also, a treatment of rice bran with only proteolytic enzymes (Alcalase or Flavourzyme) was effective to improve the protein extraction yield up to 87.6%, with a protein hydrolysis degree of 8.8%.

Lactic acid fermentation of cereals

Lactic acid fermentation can be used to increase bran protein liberation and extractability as well as to modify the composition and nutritional profile of the proteins. Cereal fermentations are one of the oldest food processes transforming flours and grains into food products, such as bread and beer. Traditionally, lactic acid fermentation of cereal flour, commonly defined as sourdough, was started by mixing flour and water, which activated the LAB and yeasts naturally present in flour. The main purpose of sourdough is to act as a leavening agent in the dough and to improve the flavour and shelf-life of bread. In bakeries, the traditional use of sourdough involves a procedure in which the microbiota is maintained by repeated propagation (backslopping), and the sourdough is then used daily in bread-baking. During the 20th century industrialism, baker's yeast superseded the technological importance of traditional sourdough due to its long fermentation procedure (Poutanen et al. 2009; Gobbetti and Gänzle 2013). Nowadays, in addition to baker's yeast, commercial starter cultures with selected LAB strains are available especially for industrial baking to achieve faster and more controlled cereal fermentations.

Generally, the lactic acid fermentation of cereals is done at moderate temperatures (e.g. from 20 °C to > 35 °C) for variable times from 3–8 h up to 5 days (De Vuyst and Neysens 2005). During spontaneous fermentation, the most competitive microbes, LAB and yeasts, will multiply and dominate the microflora (Hammes et al. 2005). The LAB present in cereal fermentations are typically a heterofermentative species of the genus *Lactobacillus*, most common being *Lactobacillus sanfraciscensis*, *Lactobacillus brevis* and *Lactobacillus plantarum* (Corsetti and Settanni 2007). Compared to LAB, the content of yeasts is significantly lower in sourdoughs: the yeast/ LAB ratio is around 1:100 (Corsetti and Settanni 2007). Up to 20 species of yeasts have been isolated from cereal fermentations, however *Saccharomyces cerevisiae*, (also used as baker's yeast), *Candida humilis*, *Saccharomyces exiguus* and *Issatchenkia orientalis* are the most typical yeasts present (De Vuyst and Neysens 2005). The microflora of cereal fermentations is influenced by the cereal flour, chemical composition and processing conditions, such as fermentation temperature, dough yield, pH, redox potential and amount and duration of backslopping (De Vuyst et al. 2014). During fermentation, the LAB metabolise the soluble carbohydrates of cereal flours (e.g. glucose and fructose) and produce lactic acid, CO2, acetic acid and/or ethanol. Furthermore, the yeasts consume glucose to produce carbon dioxide and ethanol. The acids produced by LAB gradually lower the pH below 5, which increases the solubilisation of substrates (e.g. protein and phytate) and also activates the endogenous enzymes present in flour (Gänzle 2014).
The activation of endogenous enzymes depends on the pH, varying with specific enzymes. For example, wheat β-amylase is active in range of

5.0–6.7, wheat phytases 5.0–7.2 and proteases at 3.0–6.0 (Mathewson and Seabourn 1983; Leenhardt et al. 2005; Lim and Tate 1973; Loponen 2006). In turn, the activation of the endogenous enzyme pool produces nutrients such as fermentable sugars and nitrogen sources for microbial metabolism. The combined action of endogenous enzymes, LAB and yeast modify the nutritional, technological and sensory properties of cereals.

As Poutanen et al. (2009) reviewed, fermentation improves the bioavailability of minerals and bioactive compounds, such as folate, thiamine and riboflavin in whole grain. Cereal fermentation has also been shown to reduce starch digestibility, resulting in the lower glycaemic and insulin index of sourdough breads (Juntunen et al. 2003; Maioli et al. 2008). Furthermore, fermentation has been suggested to be a potential tool in producing gluten-free products by degrading gluten proteins (De Angelis et al. 2006) and to possibly promote gut health by specific exopolysaccharide-producing LAB strains (Korakli et al. 2002).

Effect of bioprocessing on wheat bran proteins

Bioprocessing methods combining the action of hydrolytic enzymes and fermentation by commercial starters or by specific strains of LAB and yeasts have recently gained more interest as a potential method for targeted modifications in cereal materials. The effects of different bioprocessing methods on the chemical, nutritional and bread baking properties of wheat bran are summarised in Table 3.

The addition of hydrolytic enzymes can promote microbial metabolism by providing fermentable substrates, subsequently causing the faster acidification and activation of the endogenous enzymes of bran (Coda et al. 2014b). The combined action of added hydrolytic enzymes and endogenous enzymes is effective in degrading the cell walls of brans, thus improving the liberation and solubilisation of the bioactive compounds and nutrients within the layers. Nordlund et al. (2013) and Coda et al. (2014a) showed that the degradation of rye and wheat bran cell walls by bioprocessing were most evident in the sub-aleurone layer and in the endosperm side of the aleurone layer.

The breakdown of cell walls was more extensive with the addition of cell wall hydrolysing enzymes than with fermentation alone. In addition to modifications in the bran polysaccharides, the fermentation-induced activation of endogenous proteases liberates and increases bran protein solubilisation, ultimately modifying and improving their bioavailability. Nordlund et al. (2013) observed that bioprocessing by fermentation with baker's yeast in combination with Grindamyl A 1000 (α-amylase) and Depol 740L (mainly xylanase, endoglucanase and β-glucanase) partly liberated the proteins from the aleurone cells in rye bran and increased the water-soluble protein content from 32% to 53%. Coda et al. (2014b) reported a small but significant increase of soluble protein content (from 18–19% to 20–21%) in the water/salt fraction of wheat bran bioprocessed either by *Lactobacillus brevis* E95612 and *Kazachstania exigua* C81116 alone or in combination with Grindamyl and Depol 740L.

The effect of different bioprocessing methods is also evident on the protein quality due to the proteolysis taking place during fermentation. As mentioned, LAB fermentation causes acidification, which activates

Table 3. Examples of different combinations of bioprocessing methods by microbial fermentation and hydrolytic enzymes on chemical, nutritional and technological properties of wheat bran and wheat bran-enriched breads in comparison to native bran or bread with added native bran.

Bioprocessing	Effects	Reference
LAB fermentation	▪ Increased content of total phenols, antioxidant and phytase activities, content of soluble dietary fibre (SDF) in breads ▪ Improved *in vitro* protein digestibility, nutritional and glycaemic indexes in bread ▪ Improved bread properties (hardness, volume)	Pontonio et al. 2017
LAB fermentation or yeast fermentation	▪ Increased contents of total free amino acids and total phenols, phytase activity and *in vitro* protein digestibility ▪ Decreased antioxidant activity, phytic acid content and starch hydrolysis rate ▪ Improved bread texture (loaf volume, hardness, resilience, fracturability)	Rizzello et al. 2012a Leenhardt et al. 2005
LAB and yeast fermentation	▪ Increased contents of WEAX and ferulic acid, decreased content of phytic acid	Manini et al. 2014 Katina et al. 2006
Yeast fermentation with and without starch- and cell wall-hydrolysing enzymes	▪ Increased contents of protein, SDF, WEAX and free ferulic acid ▪ Decreased contents of total dietary fibre, insoluble dietary fibre and β-glucan ▪ Improved *in vitro* protein digestibility, catabolism of carbohydrates and bioaccessibility of phenolic compounds ▪ Improved bread texture (volume and hardness)	Anson et al. 2009 Hartikainen et al. 2014 Nordlund et al. 2013
LAB and yeast fermentation with and without starch- and cell wall-hydrolysing enzymes	▪ Increased antioxidant and phytase activities, solubility of AX, contents of protein, peptides and total free amino acids and *in vitro* protein digestibility ▪ Decreased content of starch ▪ Improved dough stability and bread properties (loaf volume, flavour and shelf-life)	Coda et al. 2014a, 2014b
Spontaneous or yeast fermentation with and without LAB	▪ Increased contents of folates, phenolic acids and WEAX, ▪ Improved bread texture (loaf volume, crumb structure and shelf-life)	Katina et al. 2012a Salmenkallio-Marttila et al. 2001
Xylanase or LAB and yeast fermentation	▪ Increased soluble protein content, retarded post-prandial glucose and insulin responses	Lappi et al. 2010

the endogenous enzymes. The enzymes hydrolyse proteins into peptides, which are further degraded into amino acids by the intracellular peptidases of LAB (Gobbetti and Gänzle 2013).

The hydrolysis of bran proteins can be reflected in the nutritional properties of wheat bran as shown in the studies by Nordlund et al. (2013) and Coda et al. (2014a). According to Nordlund et al. (2013) bioprocessing

with baker's yeast, Grindamyl A1000 and Depol 740L significantly improved the *in vitro* protein digestibility of rye bran, being completely digested after pepsin treatment for 180 min. In comparison, the *in vitro* digestibility of native rye bran protein was significantly lower, at 75%. Since no significant acidification of the bran occurred during fermentation, the authors concluded that the bioprocessing improved the release and solubilisation of proteins from the aleurone layer rather than degrading the proteins.

In the study by Coda et al. (2014a), in addition to utilising two bioprocessing methods (fermentation with *L. brevis* E95612 and *K. exigua* C81116 alone or in combination with Grindamyl and Depol 740L), the effect of bran particle size (from 50 μm to 750 μm) was included in evaluating the nutritional quality of the bran proteins. The results showed that the peptide content increased from 33–51 mg/kg to 43–63 mg/kg by fermentation and further to 65–80 mg/kg by fermentation in combination with enzymes as a consequence of the activation of endogenous enzymes and the proteolytic activity of microbes during the bioprocesses. Similarly, the free amino acid content increased from 2300–3000 mg/kg to 2700–3300 mg/kg by fermentation and to 4000–4400 mg/kg by bioprocessing with fermentation and enzymes. The protein hydrolysis was also reflected in the nutritional quality. The *in vitro* digestibility of the bran proteins showed a small but significant increase, from 39% to 40%, by the treatments of 400 μm sized bran with fermentation alone and was further improved to 43% when the enzymes were combined with fermentation. A similar trend was also obtained in the calculated nutritional indexes of the bioprocessed brans. Fermentation alone increased the essential amino acid (EAA) index (ratio between the EAA of test and reference protein EAA) by 5–10% and in combination with added enzymes by 7–13%. The biological value indicating the utilisable fraction of the protein was increased by 7–12% by fermentation and 11–16% by both fermentation and enzymes. Furthermore, protein efficiency ratios (protein quality based on leucine and tyrosine) and nutritional indexes (EAA and protein quantity in combined) increased by fermentation 0–8% and 5–17%, respectively, and by fermentation with enzymes 2–18% and 12–24%, respectively.

Plant proteins are also precursors of bioactive peptides, which are specific sequences of peptides encrypted on the native proteins and released by digestive proteases or by microbial and endogenous proteases activated during fermentation (Gobbetti and Gänzle 2013). Bioactive peptides can exert a positive impact on health, reducing the risks of chronic diseases or improving the digestive, immune and nervous system (Korhonen and Pihlanto 2006). The lactic acid fermentation of germ and whole wheat has been shown to liberate bioactive peptides, such as ACE-inhibitory peptides and peptides with antioxidant activities as well as a non-protein amino acid γ-aminobutyric acid (GABA) (Coda et al. 2012; Rizzello et al. 2008). These bioactive compounds potentially improve health by regulating blood pressure, lowering blood cholesterol and acting as anticarcinogenic compounds (Rizzello et al. 2012b; Udenigwe and Aluko 2012).

Technofunctional properties of plant proteins

The high demand for plant-based protein alternatives has increased the research and development of functionally comparable plant protein ingredients as potential replacements for animal-sourced protein foods. The major challenges in utilising plant proteins are the lower nutritional value compared to animal proteins and difficulties in optimising the functionality of the proteins (i.e. low solubility of the proteins) as well as the low purity of the obtained protein fractions (i.e. presence of starch, dietary fibre, lipids) (Day 2013; Sozer et al. 2017). The industrially produced wheat-based protein ingredients are manufactured mainly from the gluten fraction and used in flour fortification. Moreover, the unique technological properties of wheat gliadins and glutenins have been widely studied, whereas the water-soluble protein fractions of wheat bran are not yet well characterised.

The functional properties of proteins, such as solubility; thermal stability; emulsifying, gelling and foaming properties, affect the processing, storage and end-product quality of foods, thus determining their applicability to food systems. The molecular composition and structure of proteins regulate the functional characteristics of the individual proteins and their interaction with each other and other compounds (e.g. carbohydrates, lipids, gases, salts and water). Also, the surrounding environment, such as temperature and pH, changes the protein structure and affects the functional properties during food processing.

The solubility of proteins is a prerequisite for protein functionality, influencing other characteristics such as the emulsifying, foaming and gelling properties and behaviour in food applications (Kinsella and Melachouris 1976). In general, cereal proteins have low solubility at pH 5–7, which is in range of common food products (Sozer et al. 2017). In a study by Idris et al. (2003), wheat bran protein isolates were made by wet-alkaline extraction and isoelectric precipitation, and the technofunctional properties of the proteins were evaluated as a function of pH and salt concentration. The protein isolates had higher protein solubility, emulsifying and foaming properties (capacity and stability) in alkaline conditions than in acidic conditions. The addition of 1M NaCl improved the emulsifying and foaming capacities at pH 8. However, at pH 5.5, close to the precipitation pH of the protein isolates, the protein solubility, emulsifying capacity and foaming properties (capacity and stability) were the lowest. Furthermore, the protein isolates had comparable water- and oil-holding capacities to those of other plant proteins but no gelling properties.

Lactic acid fermentation and enzymatic treatments have been studied as a potential method to improve the technofunctional properties of plant proteins, for example, from oat bran, rice bran, lupine, hemp, and soy (Wang et al. 1999; Guan et al. 2007; Yin et al. 2008; Meinlschmidt, et al. 2016b; 2016a; Klupsaite et al. 2017; Prosekov et al. 2018). Enzymatic treatments with carbohydrases or proteases have been shown to increase the protein solubility of protein isolates when compared to isolates prepared without enzymatic treatments (Table 4). In a study by Prosekov et al. (2018) the carbohydrase pre-treatment of defatted oat bran increased the protein solubility of protein isolates by removing the concomitant starch and dietary fibres form the isolate. The carbohydrase pre-treatments have also been reported to either have no effect or to selectively improve the functional

Table 4. Examples of effects of enzymatic and microbial bioprocessing methods on the functional properties of plant-based protein concentrates and isolates.

Raw material	Treatment	End product	Protein solubility	WHC / OHC[1]	Emulsifying properties (EA /ES)[2]	Foaming properties (FA/FS)[3]	Reference
Defatted oat bran	Amyloglucosidase prior isolation	Protein isolate	+	+ / -	N/A[4]	+ / -	Prosekov et al. 2018
Rice bran	Xylanase + phytase prior to isolation	Protein isolate	+	N/A[4]	Not altered	Not altered	Wang et al. 1999
Defatted rapeseed protein isolate	Alcalase and endoprotease (3.1–7.7% DH[5])	Protein hydrolysate		+ / +	+ / +	+ / -	Vioque et al. 2000
Defatted oat bran concentrate	Trypsin (4.1, 6.4% and 8.4% DH[5])	Protein hydrolysate	+	+ / -	+ / + and - (pH dependent)	+ / -	Guan et al. 2007a
Soy protein isolate	Several proteolytic enzymes (2–13% DH[5])	Protein hydrolysate	+	-/+	+ / -	+ / -	MeinIschmidt et al. 2016b
Soy protein isolate	LAB fermentation	Fermented protein isolate	+ (pH 4), - (pH 7)	+ / +	- / N/A[4]	+/ Not altered	MeinIschmidt et al. 2016a
Defatted lupine wholemeal	LAB fermentation prior isolation of proteins	Fermented protein isolate	+		+ / -	+ / +	Klupsaite et al. 2017

1 WHC: water-holding capacity, OHC: oil-holding capacity
2 EA: emulsifying activity, ES: emulsifying stability
3 FA: foaming activity, FS: foaming stability
4 N/A = not analysed
5 DH: degree of hydrolysis

properties of isolated cereal proteins, such as water-holding capacity and foaming properties (Prosekov et al. 2018; Wang et al. 1999).

Protein hydrolysates have been increasingly studied to obtain protein and peptide products with improved technofunctional and/or bioactive properties. The controlled partial hydrolysis of plant proteins has been performed on the protein isolate, in contrast to the carbohydrase treatments done prior to protein isolation. In general, the hydrolysates have increased protein solubility in comparison to native proteins (Guan et al. 2007; Meinlschmidt 2016b). The partial hydrolysis disrupts the tight protein structure, exposing more charged and polar areas to the surface for interactions with water (Guan et al. 2007). The technofunctional properties of the protein hydrolysates have been shown to be dependent on the hydrolysis rate and pH as well as the proteolytic enzyme used. Vioque et al. (2000) reported that the lowest degree of hydrolysis by Alcalase and endoprotease (3.1%) was most

optimal in increasing whippability, foam capacity, water and oil absorption and the emulsifying properties of rapeseed protein hydrolysate. In contrast, Guan et al. (2007) obtained the highest water-holding capacity and emulsifying and foaming capacities of oat protein hydrolysate with the highest degree of hydrolysis (8.3%) in comparison to oat proteins with a lower degree of hydrolysis (0.0–6.4%). However, these properties were dependent on pH and, close to the protein pI, the hydrolysates showed the highest foaming and emulsifying capacities but the lowest stability.

Meinlschmidt et al. (2016b) noted that the changes in the technological properties of hydrolysed soy protein isolates were dependent on the proteolytic enzyme preparation used, although all protease treatments improved emulsifying and foaming capacities. In addition, the commercial enzyme preparation used influenced the sensory profile of the soy protein hydrolysate, resulting in, for example, an increased intensity of bitterness (Alcalase treatment) or a reduced intensity of astringent and beany flavours (Papain treatment). In another study, by Meinlschmidt et al. (2016a), the effect of lactic acid fermentation on the technological properties of a soy protein isolate was evaluated. The fermentation had similar effects to treatments with proteolytic enzymes, improving protein solubility, water- and oil-holding capacities and foaming capacity. Furthermore, the sensory profile of the isolate was improved with decreased bitter and beany off-flavours. Klupsaite et al. (2017) studied the effect of the pre-fermentation of lupine flour on the technofunctional properties of a lupine protein isolate. The lactic acid fermentation longer than 24 h decreased the total protein content but increased the soluble protein content in lupine. Altogether, the emulsifying and foaming properties were improved at pH 8 by the fermentation pre-treatment. In another study, by Chinma et al. (2014), spontaneous or yeast fermentation was used prior to the production of rice bran protein concentrate by alkaline extraction and protein precipitation. The pre-fermentation of rice bran increased the protein content in the concentrates from 66% to 73%. In general, the fermented rice bran protein concentrates had improved water absorption, nitrogen solubility and foaming and emulsifying properties in comparison to protein concentrate made without pre-fermentation. In particular, the yeast-fermentation improved these features to a greater degree than did spontaneous fermentation.

Plant protein isolates in bakery products

Plant-based proteins can be used in bakery products to improve nutritional quality (e.g. protein content or essential or restricting amino acids contents) or to substitute animal-based proteins, such as eggs. Although the knowledge on the technofunctional properties of plant-based protein isolates can be used as a prerequisite for food applications, significantly less is known about the actual performance of cereal-based protein isolates in bakery products. Nevertheless, some studies have been made on applying protein isolates and concentrates, such as from rice bran, soy, pea, lupine, sesame seed and sunflower meal, to breads and biscuits (Paraskevopoulou et al. 2010; Yadav et al. 2011; Ziobro et al. 2013; Majzoobi et al. 2014; Shchekoldina and Aider 2014; Chinma et al. 2015).

In a study by Paraskevopoulou et al. (2010), the supplementation of wheat flour in bread by 5% or 10% with a globulin-rich or albumin-rich

lupine protein isolate (protein content higher than 92%) had significant changes in dough rheology and baking performance. The addition of a globulin-rich protein isolate significantly increased water absorption and dough development time and stability but decreased elasticity. Similar changes in the physical properties of dough have also been reported by other authors, with breads substituted by lupine and sesame seed concentrate and isolates (substitution to the protein content of 15–20% in bread dough) (El-Adawy 1997; Mubarak 2001). Applying an albumin-rich lupine protein isolate significantly increased dough development time and stability but had no effect on dough water absorption and decreased dough elasticity only at the 5% addition level. These changes in dough rheology by the concentrates were suggested to be caused by possible interactions with gluten proteins and by the differences in protein hydration and level of water competition between the two protein types.

The addition of protein concentrates and isolates has been reported to also change the texture and sensory profile of bread. In general, breads supplemented by legume-, seed- or cereal-based concentrates or isolates have a decreased loaf volume and an increased bread hardness, gumminess and chewiness compared to wheat bread (El-Adawy 1997; Jiamyangyuen et al. 2005; Paraskevopoulou et al. 2010; Chinma et al. 2015). The changes in bread characteristics were intensified by increasing the amount of added protein concentrate or isolate. The lower volume in isolate/concentrate-enriched breads has been associated with the dilution of the gluten network (El-Adawy 1997; Chinma et al. 2015), and, to confirm the theory, Paraskevopoulou et al. (2010) added extra gluten to breads enriched with lupine protein isolates to compensate the substituted gluten. The addition of extra gluten to the level of the control wheat bread increased the loaf volume, however it still remained lower than that of the control wheat bread. The authors concluded that the smaller loaf volume in isolate-enriched breads was not only caused by gluten dilution but also by the isolate particles mechanically disrupting the gluten network. Without extra gluten, the hardness of isolate-enriched breads seemed to be more linked to the substitution level rather than to the protein type added (albumin/globulin). However, breads containing an albumin-rich lupine protein isolate had significantly lower bread firming during 24 h and 48 h storage compared to wheat bread and bread with a globulin-rich lupine protein isolate. The firming was even greater reduced when extra gluten was added to bread, indicating synergistic actions between the lupine albumins and gluten in inhibiting amylopectin retrogradation and improving water-retention capacity. To overcome the negative impacts of protein isolates on bread characteristics, Chinma et al. (2015) used spontaneous or yeast-fermentations as a pre-treatment for producing rice bran protein isolates. The addition of 10% of fermented isolates resulted in breads comparable to wheat bread in terms of sensory profile (i.e. colour, texture and flavour) and volume. Higher levels of fermented rice bran isolates (15%) resulted in significantly increased bread hardness and darker crumb and crust colour and decreased the overall scores in sensory profile (such as flavour, colour and texture). No significant differences were observed in the sensory profile between the two differently fermented rice bran protein isolates.

3 Aims of the study

Wheat bran is a potential raw material for the production of plant-based protein ingredient that is currently underutilised due to challenges in regard to the bioavailability and technological properties of the proteins. Studies of bran processing have been mainly focusing on the enzymatic and microbial modifications in the dietary fibre fraction and the process applications of whole bran in food products. However, limited research has been conducted on modifications in the bran protein fractions during bioprocessing with enzymes and/or microorganisms. Even fewer studies have been performed on the bioprocess-induced modifications in the technofunctional properties of wheat bran protein isolates. The use of lactic acid fermentation together with hydrolytic enzymes could be an efficient tool to modify the biochemical composition, bioavailability and technological properties of bran proteins. The main aim of this work was to examine bioprocessing techniques to obtain improved wheat bran protein bioavailability, nutritional quality and technological properties for food applications.

The objectives of the study were as follows:

1. To examine the role of lactic acid fermentation and exogenous and endogenous enzymes both individually and in combined action in the liberation, solubilisation and nutritional quality of wheat bran proteins (I,II).

2. To investigate how different bioprocessing methods impact wheat bran cell wall integrity and degradation (I, II).

3. To understand the effect of wheat bran bioprocessing on the biochemical and technological properties of bran protein isolates (III).

4 Materials and methods

A general outline of the materials and methods used in the study are described in this section, and the detailed descriptions can be found in the original publications, I–III. Three additional micrographs of native and bioprocessed wheat brans, the protein yield in the isolates and baking experiments are unpublished, thus the experiments are described in detail below.

4.1 Raw materials

4.1.1 Wheat bran, enzymes and microorganisms for bioprocessing

Three batches of commercial wheat brans were used as raw material in studies I–III (Table 5). In publications I and II, the bran was milled with Turborotor technology (Mahltechnik Görgens GmbH, Dormagen, Germany) and had a median particle size of 160 μm and 177 μm, respectively. In publication II, coarse bran with a median particle size of 750 μm was used.

Table 5. Particle size and chemical composition of wheat bran raw materials used in the study.

Publication	I	II	III
Producer	Fazer Mills	Fazer Mills	Lantmännen Cerealia AB
Median bran particle size	160 μm	750 μm	177 μm
Protein	18.5%	16.0%	14.0%
Dietary fibre	47.9%[1]	44.3%[1]	54.0%[1]
Starch	15.4%[1]	16.8%[1]	11.5%
Fat	4.8%[1]	4.1%[1]	6.0%[1]

1 Composition provided by the producer

The commercial enzyme preparations used for the enzymatic treatments are listed in Table 6. The enzyme activity profiles, including endo-1,4-β-xylanase at pH 6 (Bailey et al. 1992), endo-1,4(1,3)-β-glucanase at pH 6 (Zurbriggen et al. 1990), β-glucosidase at pH 5 (Bailey and Nevalainen 1981), polygalacturonase at pH 5.5 (Bailey and Pessa 1990) and neutral protease at pH 7.5 (Matsubara et al. 1958), were determined using colorimetric assays. The phytase activity of the phytase enzyme preparation was reported by the manufacturer.

Table 6. Enzyme preparations used for wheat bran bioprocessing.

Enzyme preparation	Producer	Source organism	The main activity in preparation	Measured activity (nkat/g)	Protease activity (nkat/g)	Publication
Depol 761P	Biocatalysts	*B. subtilis*	Xylanase	35,828	N.d.	I, II
Bel'ase B210	Puratos	*B. subtilis*	Xylanase	19,517	N.d.	III
Econase CE	AB Enzymes	*T. reesei*	Xylanase	28,000	N.d.	I
Econase WBP	AB Enzymes	*T. reesei*	Xylanase	126,821	N/A	I
Depol 740 L	Biocatalysts	*Humicola* spp	Xylanase	26,937	45	I
Spezyme CP	Genencor	*T. reesei*	Xylanase	35,179	3	I
Celluclast 1.5L	Novozymes	*T. reesei*	β-glucanase	26,798	N.d.	I
Glucanase 5 XL	Biocatalysts	*T. longi-brachiatum*	β-glucanase	37,735	8	I
Viscoferm	Novozymes	*Trichoderma/ Aspergillus*	xylanase / β-glucanase	15,715, 33,284 / 44,317	2.5	I, II III
Novozym 188	Novozymes	*A. niger*	β-gluco-sidase	5,908	31	I
Viscozyme L	Novozymes	*A. aculeatus*	Polygalacturonase	75,205	60	I
Corolase 7089	AB Enzymes	*B. subtilis*	Neutral protease	8,476	8,476	I
Phytase	Ultra-Biologics	*A. niger*	phytase	25,005	N.d.	III

N.d.: no activity detected
N/A: not analysed

In publication II, *L. brevis* E-95612 and *C. humilis* E-96250 from the VTT Culture Collection were used for controlled fermentation. In publication III, commercial starter culture Florapan LA4K (Lallemand, Montreal, Canada) with *L. brevis*, *L. plantarum* and *S. cerevisiae* strains were used.

4.1.2 Ingredients for bread baking

For bread baking, wheat flour was purchased from Helsingin Mylly Oy (Helsinki, Finland). The flour contained 12.5 g/100 g of protein, 70 g/100 g of carbohydrates and 2.1 g/100 g of fat, as reported by the manufacturer. The flour had a moisture content of 14.0 ± 1.0 g/100 g. Also, fresh yeast (Suomen Hiiva Oy, Lahti, Finland), shortening (Flora Culinesse, Unilever Oy, Helsinki, Finland), table salt (Jozo Salt, Denmark) and sugar (Dansukker, Suomen Sokeri Oy, Kantvik, Finland) were used in the baking.

4.2 Bioprocessing treatments

The bioprocessing of wheat bran was performed by mixing wheat bran with water (I–III) or a buffer (II) in ratios of 10:90 w/v (I), 20:80 w/v (II) or 30:70 w/v (III). All treatments, except those made by chemical acidification, were done using tap water without pH adjustment. In these treatments, the intrinsic pH was at the natural pH of bran (6.5–6.8). For treatments applying chemical acidification, the wheat bran was mixed with a lactic/acetic acid buffer (4:1 molar ratio, pH 4.5) instead of water (II). The bioprocessing methods using different combinations of added enzymes, starters and antibiotics in various temperatures, times and enzyme dosages are summarised in Table 7. The hydrolytic enzymes were dosed at 100 nkat/ g bran (I, II) or 500 nkat/g bran (III) according to their xylanase activity, except for Glucanase 5XL and Corolase 7089 (I), which were dosed (100 nkat/g bran) according to their β-glucanase and neutral protease activities, respectively. In addition, Phytase was dosed as 250 nkat of phytase/g bran (III).

The bioprocessing treatments were performed at temperatures and times ranging from 30–37 °C and 4 to 24 h, respectively. After treatments, the bran-water/buffer slurries were either lyophilised (II), centrifuged followed by lyophilisation of the supernatant (I) or the slurries were mixed with extraction solutions (II, III). All treatments had control samples designed specifically for each publication to represent the most suitable reference for the study. In publications I and II, control samples were made by mixing bran with water and lyophilising the slurry (II) or with the water-soluble fraction (I) without incubation and after incubation for 4–16 h (I). In publication III, a control bran protein isolate was produced by extracting the proteins from native bran, as described in the next section.

Table 7. Summary of wheat bran bioprocessings and treatment conditions used (time and temperature).

Bioprocess	Sample name	T (°C)	t (h)	Enzyme preparation	Starters	Anti-biotics	Chemical acidification	Publication
Exogenous enzymes (spontaneous fermentation)	according to enzyme preparation + incubation time	37	4, 6, 16	Depol 761P, Econase CE, Econase wheat bran plus, Viscoferm, Depol 740L, Spezyme CP, Celluclast 1.5L, Glucanase 5 XL, Novozym 188, Viscozyme L, Corolase 7089				I
Endogenous enzymes	End	30	24			+[1]	pH 4.5	II
Exogenous enzymes	Enz	30	24	Depol 761P + Viscoferm		+[1]		II
Endo- and exogenous enzymes	End/Enz	30	24	Depol 761P + Viscoferm		+[1]	pH 4.5	II
Controlled fermentation	St	30	24		*L. brevis* + *C. humilis*			II
Controlled fermentation + enzymatic process	St/Enz	30	24	Depol 761P + Viscoferm	*L. brevis* + *C. humilis*			II
Spontaneous fermentation + exogenous enzymes	Sp/Enz	30	24	Depol 761P + Viscoferm				II
Controlled fermentation	Str	35	8		Florapan LA4K			III
Exogenous enzymes + controlled fermentation	StrE	35	8	Bel'ase B210 + Viscoferm	Florapan LA4K			III
Exogenous enzymes + controlled fermentation	StrEP	35	8	Bel'ase B210 + Viscoferm + Phytase	Florapan LA4K			III

1 0.01% cycloheximide and 0.01% chloramephenicol

4.3 Extraction and isolation of proteins

In publication II, water/salt soluble extracts were prepared for the analysis of total solublised nitrogen, soluble protein, peptide and amino acid contents and the electrophoretic patterns of proteins. The bioprocessed bran samples were mixed with 50 mM tris-HCl (pH 8.8) according to the modified Osborne method by Weiss et al. (1993). The samples were incubated for 1 h at room temperature under stirring, after which the soluble fraction was separated by centrifugation (20,000 g, 20 min) and finally lyophilised.

In publication III, protein isolates were made after bioprocessing by alkaline extraction and isoelectric precipitation. As bioprocessing methods, lactic acid fermentation with and without the addition of cell wall-degrading enzymes were chosen based on the results obtained from studies I–II. However, as shown in study II by the electrophoretic patterns of bioprocessed bran proteins, the long 24 h treatment time with starter fermentation in combination with Depol 761P and Viscoferm caused a significant degradation of proteins. Thus, to obtain good technological functionality of bran proteins, the optimisation of the wheat bran bioprocessing conditions was undertaken as pre-experiment to obtain maximal protein solubilisation but minimal protein hydrolysis (data not shown). The optimal conditions for the bioprocessing (35 °C, 8 h, 500 nkat of xylanase/g bran) according to the experimental design were used as prior to the production of protein isolates (III). Henceforth, the term 'bioprocessed bran protein isolates' refers to wheat bran protein isolates prepared after bioprocessing. Due to the production challenges of Depol 761P by the manufacturer, the enzyme preparation was replaced by Bel'ase B210. Four protein isolates were produced: a native protein isolate without bioprocessing pre-treatment (Control) and three protein isolates bioprocessed by starters Florapan LA4K (Str), starters and cell wall-degrading enzymes Depol 761P and Bel'ase B210 (StrE) and starters, cell wall-degrading enzymes and Phytase (StrEP). The phytase addition was to chosen to determine whether it could further improve the protein solubilisation by degrading the phytic acid present in bran.

The bran proteins were extracted after bioprocessing or from native bran (control isolate) overnight with 300 mM NaOH (4 °C, bran-NaOH ratio 1:4 w/v, pH 11.5 ± 0.2), after which the solubilised proteins were separated by centrifuging (20 min, 15,500 g, 21 °C). The extracted proteins were then precipitated by adjusting the pH to 5.5 with 2 M HCl, and the precipitated proteins were collected by centrifugation. Finally, the isolated proteins were dialysed (cut-off 14,000 g/mol, 4 °C, 3 × water change) and lyophilised. The bioprocessing, extraction and isolation methods in the studies are presented in Figure 2.

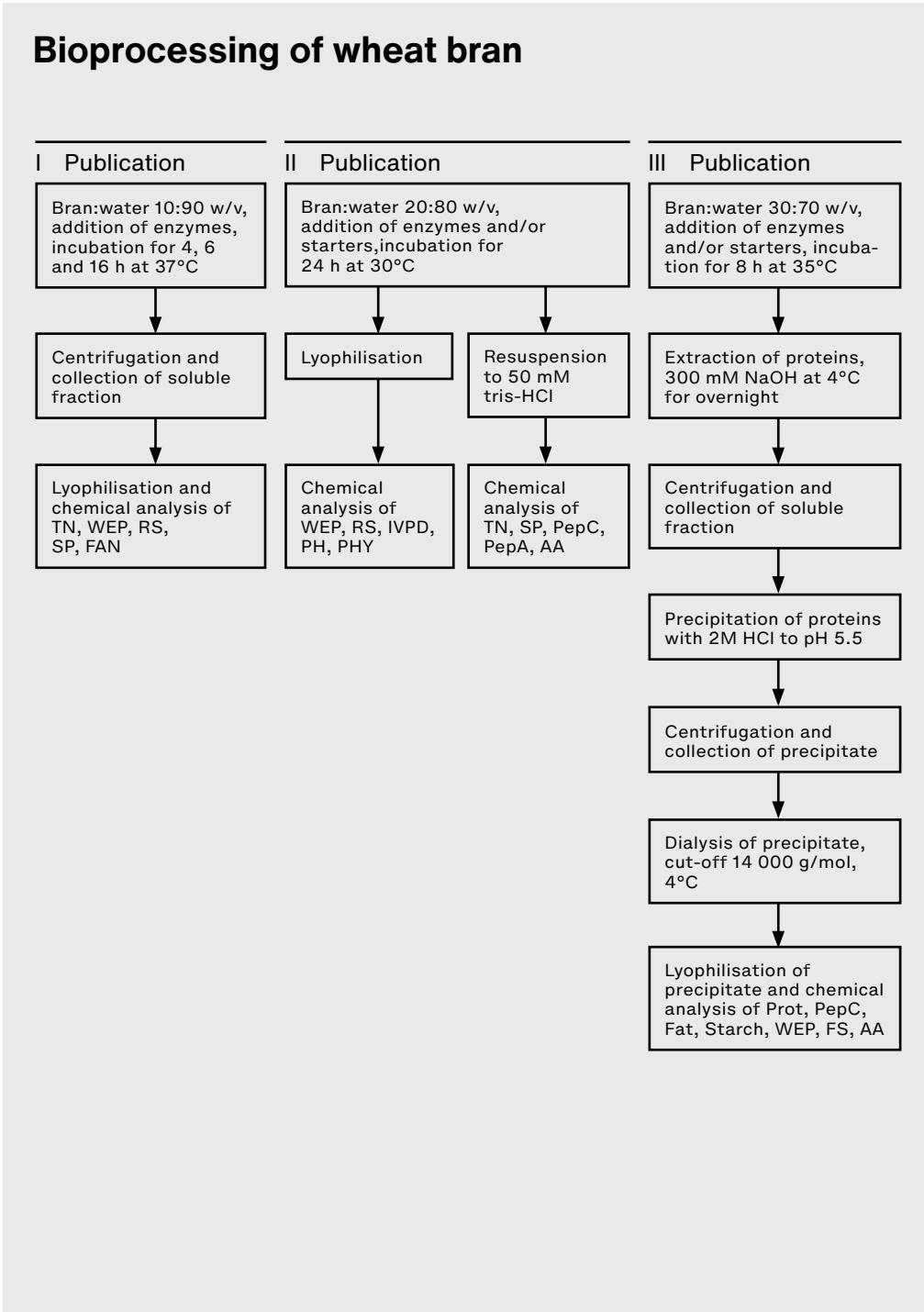

Figure 2. General flowchart of the wheat bran bioprocessing, extraction and isolation methods and chemical analysis of samples in publications I–III. TN: total solubilised nitrogen content, WEP: water-extractable pentosan content, RS: reducing sugar content, SP: soluble protein content, FAN: free amino nitrogen content, IVPD: *in vitro* protein digestibility, PH: total phenol content, PHY: phytase activity, PepC: peptide content, PepA: peptides area, AA: total amino acid content, Prot: protein content, FS: free sugars

4.4 Analytical methods

4.4.1 Chemical composition and bran structure (I–III)

The chemical compositions of the water- (I) and water/salt-soluble fractions (II) and protein isolates (III) were determined to evaluate the chemical changes during bioprocessing. The content of the total nitrogen of the samples was analysed by the Kjeldahl (I, II) or Dumas combustion method (III). The total solubilised nitrogen content after treatments was calculated as the percentage of the solubilised nitrogen in the total nitrogen content in the bran (I, II). In publication III, the total protein content in the protein isolates was calculated using a conversion factor of 6.31 from nitrogen to protein, as recommended for wheat bran by the FAO (2002).

To evaluate the hydrolysis of cell wall structure during bioprocessing, the contents of water-extractable (WE) pentosans (I–III) and reducing sugars (I–II) were analysed using the colourimetric phloroglucinol method (Santala et al. 2011) and the dinitrocalicylic method (Bernfeld 1955). In addition, starch and free sugar contents were determined in the wheat bran protein isolates (III). The content of total starch was analysed spectrophotometrically by AACC method no. 76–13, and the free sugar content as combined amounts of glucose, saccharose, fructose and maltose was analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The fat content of the bran protein isolates was analysed using the gravimetric method (III).

Fluorescence microscopy was used to evaluate the effect of enzymatic and bioprocessing treatments on wheat bran cell wall integrity and protein liberation. The method was performed according to Rosa et al. (2013) with small modifications. The sections cut from the samples were stained with Calcofluor and acid fuchsin to visualise cell wall β-glucan and protein, respectively. The microstructure of the following samples was visualised as presented in study I: wheat brans incubated for 6 and 16 h without any enzyme addition and brans treated with Depol 761P, Viscoferm and Corolase for 6 h. In addition to the visualisation of native bran, the following samples from study II were analysed: brans treated with both Depol 761P and Viscoferm at pH 6.5 for 24 h (End/Enz) and bran fermented with *L. brevis* E-95612 and *C. humilis* E-96250 in combination with Depol 761P and Viscoferm (St/Enz). The microscopic images of the samples from study II have not been published and are thus presented only in this thesis.

4.4.2 Protein quality (I, II)

The soluble protein (II) and peptide contents (II, III) were analysed spectrophotometrically using the Bradford assay and o-phtaldialdehyde method, respectively. The free amino acid content was determined by ion-exchange chromatography with a Na-cation-exchange column (II). The electrophoretic pattern and MW distribution of the wheat bran proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions (Laemmli 1970). In study I, the lyophilised water-soluble protein fractions were mixed with an SDS sample buffer (pH 8.5) and pipetted according to their protein content (70 μg protein/well) to NuPage Bis-Tris 10% minigels. In study II, the water/salt-soluble bran

protein fractions were mixed with an SDS sample buffer (pH 8.5) in a ratio of 1:1 (v/v) and pipetted to NuPage Bis-Tris 12% minigels. The gels were stained with Coomassie blue overnight, after which the gels were destained with water.

To evaluate the nutritional quality of the solubilised bran proteins, *in vitro* protein digestibility (II) was determined based on the method by Akeson and Stahmann (1964). The *in vitro* digestibility of the proteins was calculated as the percentage of the solubilised protein after enzyme hydrolysis by pepsin and pancreatin.

4.4.3 Protein yield and technofunctional properties of wheat bran protein isolates (III)

A series of experiments were performed to evaluate the technological functionality of the wheat bran protein isolates and the full description and results of the analysis can be found in study III. For this thesis, the protein yield was calculated as the percentage of protein obtained in the isolates from the total protein content in the bran.

The protein solubility of the wheat bran protein isolates was determined in a pH range of 4–8. The lyophilised protein isolates were suspended (2h) into a citric acid buffer (pH 4 and 5) or sodium phosphate buffer (pH 6, 7 and 8) in a concentration of 10 mg/ml. The percentage of solubilised nitrogen from the total nitrogen content in isolates was analysed by the Dumas combustion method.

The foaming properties of the bran protein isolates were determined using Dynamic Foam Analyser DFA100 (Krüss GmbH, Hamburg, Germany), where 100 ml (0.3 l/min) of air was conducted to 25 ml of a bran protein isolate solution (0.2% w/v in potassium phosphate buffer, pH 7.4). The foam height and foam stability were measured for 30 min using light transmission through the column. Simultaneously, the evolution of the foam structure was recorded using the Foam Structure Module attached to the column at a height of 55 mm. Images of the foam were taken every 5 min over a 30 min following time.

4.4.4 Bread baking process and analysis of bread texture

The technological potential of bioprocessed wheat bran protein isolates in food applications was evaluated from isolate-enriched wheat breads. Control, Str and StrE isolates were added to wheat breads according to their protein content, substituting 20% of the total energy by proteins from the isolates to obtain EU Nutrition and Health Claim 'high protein' (EU 2006). For a standard, wheat bread was made without the addition of isolates (wheat bread). The bran protein isolates were added to bread at a substitution level of 12.2% of the flour weight (control isolate) or 9.9% of the flour weight (Str and StrE) based on their protein content. Since the protein isolates varied in fat content, the amount of added fat was adjusted to keep a constant level of fat in the dough recipe (3.1g/100 g dough). The optimal water content in the wheat bread was determined by farinograph measurement and used for all breads. The bread recipes are presented in Table 8.

Table 8. Bread recipes for wheat breads with added bioprocessed wheat bran protein isolates. The ingredients are reported as % of flour weight (f.w.).

	Wheat bread	Control isolate bread	Str isolate bread[1]	StrE isolate bread[2]
Wheat flour	100	87.8	90.1	90.1
Protein isolate	–	12.2	9.9	9.9
Water	60.3	60.3	60.3	60.3
Yeast	5.0	5.0	5.0	5.0
Sugar	2.0	2.0	2.0	2.0
Salt	1.5	1.5	1.5	1.5
Fat	5.0	2.6	2.7	2.5

1 Str: starter fermentation by Florapan LA4K
2 StrE: starter fermentation by Florapan LA4K with Bel'ase B210 and Viscoferm

The doughs were prepared by mixing the ingredients for 3 min at a slow speed and 4 min at a fast speed with spiral mixer (Diosna SP 12F, Dierks & Söhne, Osnabrück, Germany). A floor time of 15 min at 35 °C (75 RH%) was used for the doughs (Lillnord, Topline 930, Germany), after which they were divided into 200-g loaves and moulded with a long moulder (5mm gap) (Frilado 2, Germany). The loaves were proofed in pans for 45 min at 35 °C (75 RH%) and baked at 200 °C for 15 min, beginning with 15 s of steam (Sveba Dahlen S400, Sweden). The breads were cooled for 1 h before being weighed and stored in plastic bags for textural analyses performed the next day.

The loaf volume was determined using a VolScan Profiler 300 (Stable Micro Systems, Godalming, UK) and the specific loaf volume was calculated by dividing it by the corresponding loaf weight (AACC method no. 2013). The bread structure was assessed after one and four days of baking by Texture profile analysis (TPA) using a TPA Analyzer (Stable Micro systems, UK). The breads were cut into 25-mm-thick slices, and six slices from the centre of the breads were measured for the mechanical characteristics. A TPA test was used to analyse the bread hardness and adhesiveness using a probe SMS P/36 (35 mm diameter), 5 kg load cell, 40% penetration depth and test speed of 2 mm/sec. The hardness of the breads was presented as Force (g) and adhesiveness as Force × time (g × s). Springiness is a unitless value of the ratio of the change in the original height by compression.

4.5 Statistical analysis

All bran bioprocesses were made in duplicate, and each analysis was made at least in duplicate, meaning that the results were calculated as means of at least four analysis results. However, in publication III, the fat, starch and free sugar compositions of protein isolates were calculated as means of two analysis results. The data were analysed by one-way ANOVA using the statistical software IBM SPSS Statistics, version 22. A pair-comparison of the analysis means was done by Tukey's test to test the significance ($p < 0.05$) between the replicates. When the response differed significantly ($p < 0.05$), it was indicated with a different letter.

5 Results

In the first study, 11 commercial carbohydrase and protease-active enzymes were screened for bran cell wall degradation and subsequent increase in protein solubilisation. The protein solubilisation was determined after 4–16 h of incubation, and the protein MW distribution was evaluated from the solubilised proteins. The contents of WE pentosans and reducing sugars were analysed as indicators of the bran cell wall hydrolysis during short incubation times (< 6 h). Based on the results obtained from WE pentosan and reducing sugar contents, Depol 761P and Viscoferm were chosen for the following study for bran cell wall degradation. In the second study, six different combinations of treatments by exogenous and endogenous enzymes and spontaneous and controlled fermentations were performed to evaluate their individual and combined roles in wheat bran modifications. An evaluation of the changes in bran protein quality was made, and the results are discussed in detail below. In the third study, wheat bran was bioprocessed with controlled fermentation with or without the addition of cell wall-degrading enzymes and phytase in optimal conditions aiming at maximal protein solubility with minimal proteolysis. To study the techno-functional properties of the bioprocessed proteins, the proteins were isolated by alkaline extraction followed by isoelectric precipitation. Technological properties, such as the protein solubility and foaming properties of the differently bioprocessed bran protein isolates were analysed. Finally, bread volume and texture were evaluated from wheat breads, in which 20% of the total energy was substituted by proteins from bioprocessed protein isolates.

5.1 Impact of bioprocessing on bran proteins

5.1.1 Protein solubilisation (I, II)

A comprehensive evaluation was made of enzymatic treatments on modifying bran structure and improving protein solubilisation from the bran matrix. Without any enzyme addition, only 13% (II) and 14% (I) of the nitrogen was solubilised by soaking and centrifuging (time 0 h) from coarse and fine bran, respectively. Incubating bran for 4, 6 and 16 h without enzyme addition, the nitrogen solubilisation increased to 27%, 31% and 43%, respectively (Table 9).

In total, 11 commercial carbohydrate- or proteolytic-active enzyme preparations were studied to improve wheat bran protein solubilisation (I).

Using enzyme preparations with xylanase or β-glucanase as the main activity, with very low or no protease side activity, the nitrogen solubilisation of fine or coarse wheat bran was not significantly improved during the 4–24 h treatment time in comparison to bran incubated without enzyme addition. In these treatments, the solubilised nitrogen content varied between 28–32%, 30–34% and 42–48% after 4, 6 and 16 h of incubation, respectively.

Table 9. Solubilised organic nitrogen content (%) of wheat bran bioprocessed with different combinations of commercial enzymes, endogenous enzymes and spontaneous and/or controlled fermentation (I, II).

Bioprocess	Enzyme treatment	pH adjustment/ antibiotics / starters	Solubilised nitrogen (%) Time 0 h[2]	4 h[1]	6 h[1]	16 h[1]	24 h[2]	Publication
No enzyme addition	Control		14±1	27± 2	31 ±0	43±1		I, II
Xylanase	Depol 761P, Econase CE, Econase WBP, Depol 740L, Spezyme CP			28 ± 2	32 ± 2	44 ± 2		I
β-glucosidase	Novozym 188			45 ±0	45 ±1	57 ± 1		I
Polygalact-uronase	Viscozyme L			33 ± 1	37 ± 2	48 ± 5		I
Neutral protease	Corolase 7089			58 ± 2	64 ± 1	64 ± 2		I
β-glucanase	Celluclast 1.5L, Glucanase 5 XL			30 ± 2	31 ± 1	46 ± 2		I
Xylanase + β-glucanase	Viscoferm			31 ± 0	31 ±1	45 ±4		I
Xylanase + β-glucanase	Depol 761P and Viscoferm						34 ± 1	II
Xylanase + β-glucanase	Depol 761P and Viscoferm	pH 6.5					39 ± 0	II
Endogenous enzymes + xylanase + β-glucanase	Depol 761P and Viscoferm	pH 4.5, antibiotics					63 ± 1	II
Endogenous enzymes	–	pH 4.5, antibiotics					75 ± 3	II
Controlled fermentation	–	starters[3]					52 ± 0	II
Controlled fermentation + xylanase + β-glucanase	Depol 761P and Viscoferm	starters[3]					52 ± 3	II

1 Fine bran (160 µm), treatment temperature 37 °C
2 Coarse bran (750 µm), treatment temperature 30 °C
3 *L. brevis* E-95612 and *C. humilis* E-96250

Without control of the initial pH, the pH was between 6.0–6.8 in all treatments after 4–6 h. After long incubation times, for 16 h and 24 h, the pH decreased to 3.7–4.3. To exclude the impact of the spontaneous fermentation and acidification of the wheat bran, an enzymatic treatment of the bran was performed in a combined action with xylanase (Depol 761P) and β-glucanase (Viscoferm) at pH 6.5 with the addition of antibiotics (II). This treatment did not further improve the solubilisation of organic nitrogen in comparison to the same treatment without pH adjustment.

The enzyme preparations with protease side activities, Novozym 188 and Viscozyme L, significantly increased the solubilised nitrogen content to 45% and 45%, and to 33% and 37% after 4 and 6 h of incubation, respectively. A longer treatment time of 16 h with Novozym 188 further improved the solubilisation of nitrogen, up to 57%. However, the highest solubilisation of nitrogen was obtained by a treatment with a protease-active enzyme, Corolase 7089. After only 4 h, 58% of the organic nitrogen was solubilised, and the content was increased up to 64% after 6 h. Nevertheless, a longer incubation time (16 h) did not further improve the nitrogen solubilisation.

In study II, six different combinations of enzymatic and microbial treatments were studied to elucidate the individual roles of endogenous enzymes and lactic acid fermentation on wheat bran protein solubilisation. The highest nitrogen solubilisation, 75%, was obtained by a treatment where solely endogenous enzymes were activated by chemical acidification (pH 4.5) and where microbial growth was inhibited by antibiotics. A considerable amount of solubilised nitrogen (64%) was also obtained with the combined action of endogenous enzymes and added cell wall-degrading enzymes Depol 761P and Viscoferm. A wheat bran treatment with lactic acid fermentation either with or without cell wall-degrading enzymes led to 52% of the organic nitrogen being solubilised.

5.1.2 Hydrolysis and *in vitro* digestibility of bioprocessed bran proteins (II)

To evaluate the hydrolysis degree of bioprocessed wheat bran, the soluble protein, peptide and free amino nitrogen contents of the bran soluble fractions were analysed (II). All bioprocessing treatments increased the soluble protein content, free amino acid content and *in vitro* digestibility of proteins (Table 10). In general, the enzymatic treatments for 24 h without microbial fermentation (End, Enz, End/Enz) had a higher content of soluble proteins and peptides but a lower content of free amino acids compared to bioprocessing treatments applying microbial fermentation. No difference was found in the content of soluble proteins (11.3–11.7 mg/ml) and peptides (15.0–17.7 mg/ml) between the enzymatic treatments. However, the content of free amino acids (2,588 mg/kg bran) and *in vitro* protein digestibility (25.3%) was significantly higher in the treatment with solely exogenous enzymes (Enz) in comparison to the other enzymatic treatments, End and End/Enz.

Bioprocessing treatments with either controlled or spontaneous fermentation resulted in a lower content of soluble proteins but a higher content of amino acids and *in vitro* protein digestibility in comparison to the enzymatic treatments. Among the fermented brans, starter fermentation with Depol 761P and Viscoferm contained the lowest amount of soluble proteins

(5.8 mg/ml) but had highest free amino acid content (4,391 mg/kg bran) and protein digestibility (39.1%). Fermentation without hydrolytic enzymes resulted in a significantly higher content of soluble proteins (8.1 mg/ml) but significantly lower content of free amino acids (3,477 mg/kg bran) and protein digestibility (32.7%) compared to St/Enz. The spontaneous fermentation with hydrolytic enzymes (Sp/Enz) had a content of peptides similar to that of the enzymatic treatments (17.2mg/ml) and the lowest free amino acid content and protein digestibility between the fermented brans.

Table 10. Contents of soluble protein, peptide and free amino acids and *in vitro* digestibility of bioprocessed wheat bran (II).[1]

Sample	Bioprocess	Soluble protein content (mg/ml)[3]	Peptide content (mg/ml)	Free amino acid content (mg AA / kg bran)	*In vitro* protein digestibility (%)
Control	No bioprocessing	3.3a	6.9a	1,954a	14.6a
End	Endogenous enzymes	11.7d	15.2b	2,148b	20.0b
Enz	Exogenous enzymes[2]	11.3d	17.7b	2,588c	25.3c
End/Enz	Endogenous and Exogenous enzymes[2]	11.7d	15.0ab	2,309b	18.4b
St	Starter fermentation	8.1c	13.2ab	3,477e	32.7e
St/Enz	Starter fermentation + exogenous enzymes[2]	5.8b	13.4ab	4,391f	39.1f
Sp/Enz	Spontaneous fermentation + exogenous enzymes[2]	6.4b	17.2b	2,953d	28.7d

1 Results with the same letter within each group and column have no statistical difference ($p>0.05$). Values with a differing letter have significant difference ($p < 0.05$).
2 Depol 761P and Viscoferm
3 Values are corrected for this thesis and differ from the original publication II.

5.1.3 Electrophoretic patterns of bioprocessed bran proteins (I, II)

The electrophoretic patterns of the soluble bran proteins were characterised to compare the changes in MW distribution after enzymatic and microbial bioprocesses. In Figure 3, the water-soluble proteins of wheat bran incubated for 4, 6 and 16 h without and with the addition of Corolase 7089 (neutral protease), Depol 761P (xylanase) and Viscoferm (xylanase and β-glucanase) are presented (I). Bran proteins with an MW from under 20,000 to 160,000 g/mol were detected from the SDS-PAGE gels, where the most intensively stained protein bands had an MW of 15,000 to 70,000 g/mol. In the treatments without enzyme addition and with the addition of Depol 761P and Viscoferm, the protein profiles were very similar after

4 and 6 h of incubation. After 16 h of incubation, protein bands, especially with an MW above 25,000 g/mol, were disappeared or observed less visible in these treatments, showing significant proteolysis. In the treatment with the neutral protease Corolase 7089, almost all proteins with an MW higher than 40,000 g/mol disappeared after only 4 h, demonstrating a significant degradation of proteins by the enzyme.

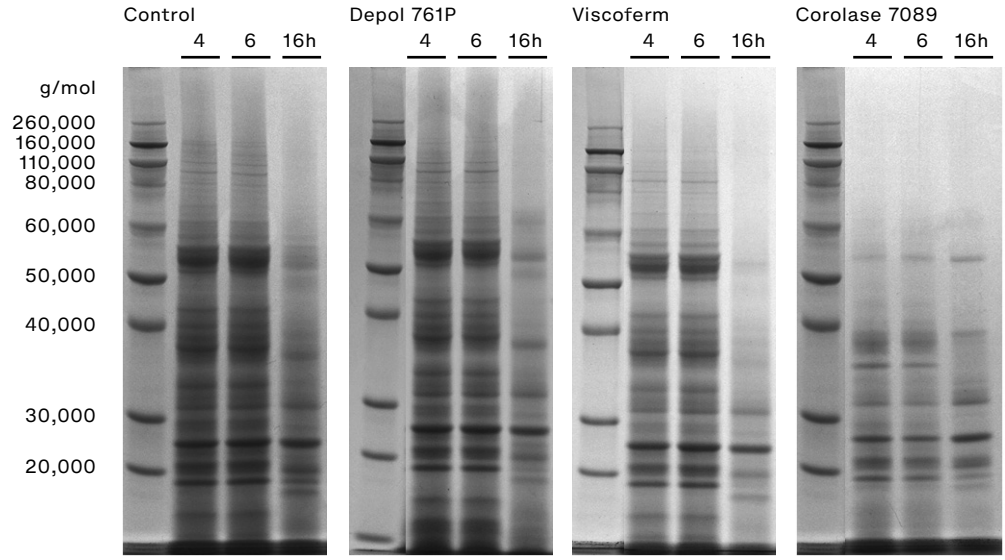

Figure 3. Electrophoretic patterns of water-soluble polypeptides of wheat bran incubated for 4, 6 and 16 h without enzyme addition (control), and with the addition of Corolase 7089, Depol 761P and Viscoferm (I).

Figure 4 presents the electrophoretic pattern of soluble bran proteins after treatments with different combinations of endogenous enzymes, added enzymes (Depol 761P and Viscoferm) and spontaneous or controlled fermentation (II). In all treatments, the protein bands, especially those with a low MW (18,000 to 32,000 g/mol), were more visible than in the control bran. Despite the protein bands being overall more intensively stained in the treatment solely with Depol 761P and Viscoferm (Enz), the protein profiles seemed to be very similar between the enzymatically treated samples (End, Enz, End/Enz). Overall, the proteins with an MW of 30,000–50,000 g/mol were less intensively stained in the fermented brans in comparison to the enzymatic treatments. Starter fermentation together with Depol 761P and Viscoferm caused a significant disappearance of bran proteins with an MW higher than 30,000 g/mol, showing their hydrolysis during the treatment.

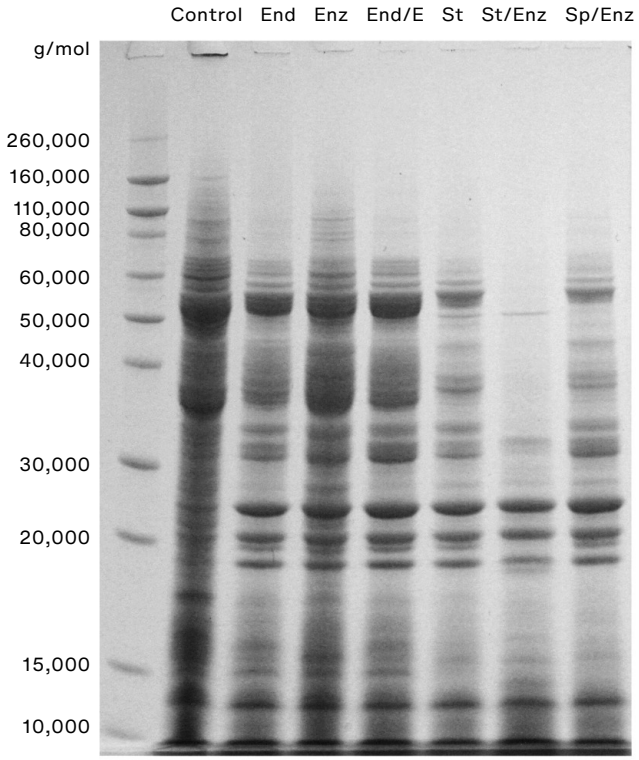

Figure 4. Electrophoretic patterns of water/salt-soluble polypeptides of control and bioprocessed wheat bran (24 h) (II). Control, without bioprocessing (time 0 h); End, endogenous enzymes; Enz, added enzymes (Depol 761P and Viscoferm); End/Enz, endogenous and added enzymes; St, controlled fermentation with starters; St/Enz, controlled fermentation with starters and added enzymes; Sp/Enz, spontaneous fermentation with added enzymes.

5.2 Effects of bioprocessing of cell wall integrity

5.2.1 Contents of WE pentosans and reducing sugars (I–III)

The contents of WE pentosans and reducing sugars were analysed to evaluate the degradation of wheat bran cell walls in bioprocessing within short incubations. The contents of WE pentosans were chosen to be analysed as representing the hydrolysis products from AX. Likewise, the content of reducing sugars was chosen to represent the hydrolysis products derived from polysaccharides such as β-glucan and cellulose. Without any enzyme addition, the contents of WE pentosans and reducing sugars increased during the first 4 and 6 h of incubation, respectively, and decreased with longer incubation times (Table 11). Thus, the microbial activity of LAB and yeast in the spontaneous and controlled fermentations partly neutralised the effect of carbohydrases by consuming the carbohydrates.

Table 11. WE pentosan content (%) of wheat bran bioprocessed with different combinations of commercial enzymes, endogenous enzymes and spontaneous and/or controlled fermentation (I, II).

Bioprocess	Enzyme preparation	pH adjustment/ antibiotics / starters	WE pentosan content (%)				Reducing sugars (g/l)				Publication
			Time				Time				
			4 h[1]	6 h[1]	16 h[1]	24 h[2]	4 h[1]	6 h[1]	16 h[1]	24 h[2,4]	
No enzyme addition			9 ± 0	6 ± 0	6 ± 0		7 ± 1	9 ± 0	3 ± 0		I, II
Xylanase	Econase CE, Econase WBP, Depol 740L, Spezyme CP		8 ± 1	9 ± 1	9 ± 1		12 ± 1	14 ± 3	7 ± 1		I
β-glucosidase	Novozym 188		6 ± 0	6 ± 0	4 ± 1		14 ± 0	14 ± 0	10 ± 1		I
Polygalacturonase	Viscozyme L		13 ± 1	15 ± 0	11 ± 1		18 ± 0	18 ± 3	20 ± 0		I
Neutral protease	Corolase 7089		6 ± 1	7 ± 1	6 ± 1		13 ± 1	14 ± 2	8 ± 3		I
β-glucanase	Celluclast 1.5L, Glucanase 5 XL		11 ± 0	12 ± 0	9 ± 0		15 ± 1	16 ± 1	10 ± 1		I
Xylanase	Depol 761P		16 ± 1e	16 ± 0	15 ± 1						I
xylanase + β-glucanase	Viscoferm		11 ± 0d	12 ±0	12 ± 0		19 ± 0	18 ± 2	15 ± 1		I
xylanase + β-glucanase	Depol 761P and Viscoferm					3 ± 0				25 ± 2	II
xylanase + β-glucanase	Depol 761P and Viscoferm	pH 6.5				4 ± 0				37 ± 2	II
Endogenous enzymes + xylanase + β-glucanase		pH 4.5, antibiotics				5 ± 0				46 ± 1	II
Endogenous enzymes		pH 4.5, antibiotics				2 ± 0				30 ±1	II
Controlled fermentation		Starters[3]				1 ± 0				2 ± 0	II
Controlled fermentation + xylanase + β-glucanase	Depol 761P and Viscoferm	Starters[3]				3 ± 0				3 ± 0	II

1 Fine bran (160 µm), treatment temperature 37 °C
2 Coarse bran (750 µm), treatment temperature 30 °C
3 *L. brevis* E-95612 and *C. humilis* E-96250
4 Values are corrected for this thesis and differ from the original publication II.

The content of WE pentosans during the incubation times was observed to be rather similar among most of the bioprocessed wheat brans. Compared to the control bran, the addition of Depol 761P, Viscoferm and Viscozyme L

enzymes significantly increased the WE pentosan content in all incubation times (4–16 h) (I). Also, with longer incubation time (24 h), the combined action of Depol 761P and Viscoferm with and without the activation of endogenous enzymes resulted in a significantly higher content of WE pentosans (3–5%) compared to the native bran (1%) (II). The other enzymatic bran treatments for 4–16 h increased the WE pentosan content to less extent or even contained less amounts of WE pentosans after the examined incubation time (I). With controlled fermentation or the activation of solely endogenous enzymes for 24 h, the WE pentosan content was 1–2% and similar to that of the control treatment (II).

The content of reducing sugars in control bran followed the same trend as the WE pentosans by increasing during the first 6 h of incubation and decreasing with longer incubation time. Compared to the control incubation without enzyme addition (7 g/l), all enzyme treatments increased the reducing sugar content to 12–19 g/l after 4 h of incubation (I). The highest increase was obtained by Viscoferm treatment (19 g/l). After 6 h of treatment, the bran with the addition of Viscoferm, Spezyme CP, Glucanase 5 XL and Viscozyme L had a significantly higher content of reducing sugars (17–18 g/l) in comparison to the control (9 g/l). With the long 16 h incubation, most of the enzyme treatments had a lower content of reducing sugars compared to shorter incubation times due to microbial activity in the samples. However, a small increase was observed with the treatment of Viscozyme L (20 g/l) (I). The reducing sugar content was significantly higher in treatments for 24 h with solely enzymes, where spontaneous fermentation was inhibited by antibiotics. Treatments either by endogenous enzymes or by exogenous enzymes, Depol 761P and Viscoferm, significantly increased the reducing sugar content up to 30 g/l and 37 g/l, respectively. However, the highest reducing sugar content (46 g/l) was obtained by the treatment with both endogenous and exogenous enzymes (End/Enz). As expected, the microbial activity during the bioprocesses decreased the content of reducing sugars in the samples. The spontaneous fermentation with Depol 761P and Viscoferm still contained a significantly high content of reducing sugars (25 g/l) compared to the control treatment (1.4g/l) although the content was significantly lower compared to solely enzymatically treated brans. The controlled fermentation with starters either with or without Depol 761P and Viscoferm had contents of the reducing sugars (1.6 g/l in St and 3.4 g/l in St/Enz) more similar to those of the control bran.

5.2.2 Microstructure of wheat bran (I)

To further clarify the effects of enzymatic treatment on the structure of wheat bran, micrographs were taken from different brans. As controls, native bran and bran incubated for 6 h and 16 h were analysed for comparison of bioprocessing methods. Wheat bran treated for 6 h with Depol 761P and Viscoferm were chosen to be analysed due to their clear impact on WE pentosan content and reducing sugar content, respectively. Also, the effect of the solely cell wall-degrading enzymes Depol 761P and Viscoferm after 24 h (Enz) and together with lactic acid fermentation after 24 h (St/Enz) were evaluated. Corolase 7089 treated wheat bran (6 h) was chosen for analysis due to its effectiveness in significantly increasing the solubilised nitrogen.

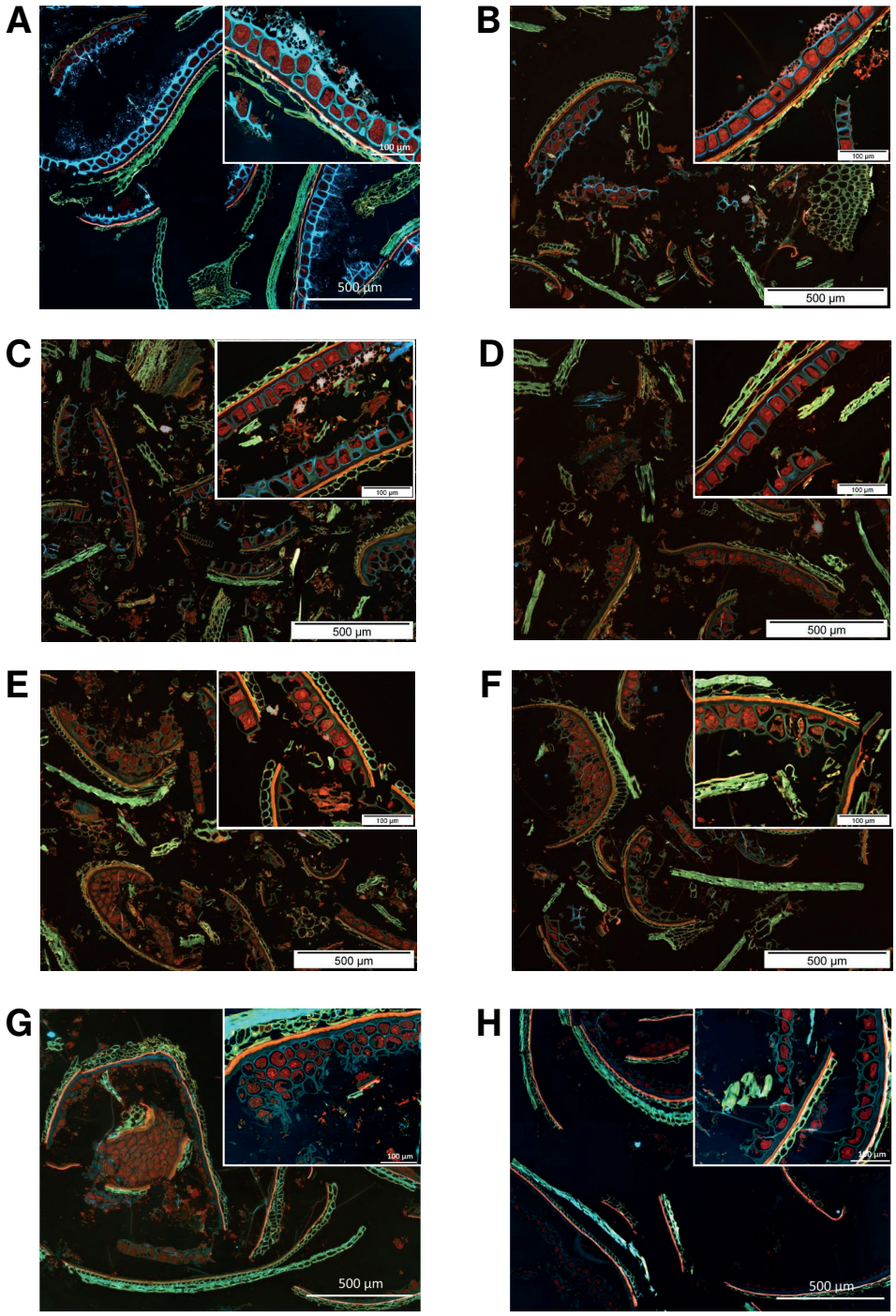

Figure 5. Microstructure of A) native coarse wheat bran, wheat bran incubated for B) 6 h, C) 16 h without enzyme addition and treatments with D) Depol 761P, E) Viscoferm and F) Corolase 7089 after 6h of incubation, and G) Depol761P and Viscoferm at pH 6.5 after 24 h, and H) Controlled lactic acid fermentation with Depol 761P and Viscoferm after 24 h. Sections from samples were stained with Calcofluor and acid fuchsin, showing cell wall β-glucan as blue and proteins as red/reddish-brown. In addition, due to autofluorescence, the pigment strand is shown as orange and the pericarp layer as light green and yellowish.

Partial liberation of aleurone protein was observed with the native bran after 6 h of incubation, shown as partially empty aleurone cells in the images (Figure 5). However, a significant amount of aleurone cells were still intact with the proteins inside the cells. Incubation for 16 h intensified the liberation of bran proteins from aleurone cells, shown as partial or even complete liberation of aleurone cells. Wheat brans treated with Depol 761P (Figure 5D) and Viscoferm (Figure 5E) either separately (including spontaneous fermentation) or combined with the pH adjusted at 6.5 (with addition of antibiotics) had most of the endosperm remains released as separated particles in the analysed sample, but the proteins inside the aleurone cells were intact. In addition, modifications were detected in the bran aleurone cell walls. In the treatment with Depol 761P, the Calcofluor staining was less intensive on the aleurone side of the cell walls, whereas in treatments with the addition of Viscoferm (Figure 5E), the staining was vague throughout the aleurone cell walls. In the treatment with the combined action of these enzyme preparations without spontaneous fermentation (24 h), the aleurone cell walls were deformed or disrupted especially on the endosperm side of bran, and partially empty cells were detected.

The treatment of bran with Corolase 7089 clearly hydrolysed the endosperm proteins since they were not detected as attached in the aleurone layer nor as separated particles in the sample. However, the proteins within the aleurone cell walls remained intact.

The most significant changes in the microstructure of wheat bran were detected in the bran with controlled fermentation together with the cell wall-hydrolysing enzymes Depol 761P and Viscoferm after 24 h (Figure 5H). The cell walls of the aleurone layer were thinner and stained less intensively. Significantly more aleurone cells were deformed and disrupted from the endosperm side of wall in comparison to the other bran samples. The proteins within the aleurone cells had empty space around them, or the proteins were partly or completely disappeared from the cells. Also, parts of endosperm were present in the samples as separated particles.

5.3 Technofunctional properties of bioprocessed wheat bran proteins

To evaluate the technological potentiality of bioprocessed wheat bran proteins, the protein solubility and foaming properties of the isolated proteins were studied (III). In addition, wheat breads with the addition of wheat bran protein isolates were produced, and the effect of bran protein addition to bread characteristics was analysed. The produced wheat bran protein isolates had a protein content of 67% (control) or 80–82% (bioprocessed), with the rest being fat (12–23%) and carbohydrates (3–16%). The protein yield in the protein isolates was 45 ± 1%, 44 ± 1%, 48 ± 0% and 46 ± 2% in the control, Str, StrE and StrEP isolates, respectively, having no significant difference between the control and bioprocessed protein isolates

($p > 0.05$). The detailed description of the chemical composition and biochemical characteristics of the bran protein isolates is found in study III.

5.3.1 Protein solubility of isolates (III)

The protein solubility of bran protein isolates was analysed from aqueous solutions of isolates at a pH range from 4 to 8 (Figure 6). In all protein isolates, the protein solubility was higher in alkaline in comparison to acidic conditions. Overall, the control isolate made without any bioprocessing pre-treatment had a higher protein solubility at a pH below 7 when compared to the bioprocessed protein isolates. The protein solubility of the control isolate increased gradually from 25% to 33% with increasing pH. The bioprocessed bran protein isolates had a protein solubility of 22–23% at a pH range of 4–6 and increased sharply with pH increasing above 6. The isolate bioprocessed with starters, cell wall-degrading enzymes (Bel'ase B210 and Viscoferm) and Phytase (StrEP) had the most significant improvement of protein solubility, to 36% at pH 8.

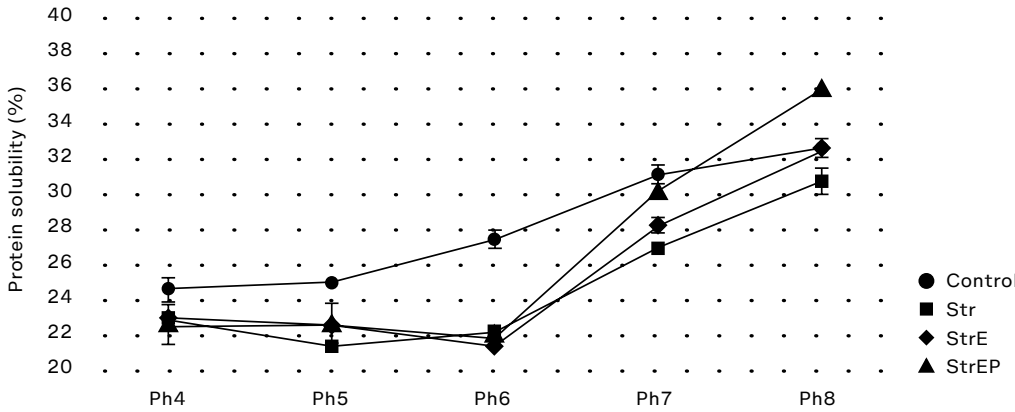

Figure 6. Protein solubility in pH 4–8 of the control wheat bran protein isolate and the isolates produced after bioprocessing. Control, protein isolate produced without bioprocessing; Str, protein isolate bioprocessed with starters (Florapan LA4K);, StrE, protein isolate bioprocessed with starters and cell wall-degrading enzymes Bel'ase B210 and Viscoferm; StrEP, protein isolate bioprocessed with starters, cell wall-degrading enzymes and Phytase.

5.3.2 Foaming properties (III)

The foaming properties of the wheat bran protein isolates were evaluated from an aqueous protein isolate solution (pH 7.4). Even though differences were not found in foam height between the control protein isolate and bioprocessed protein isolates, the foam stability was improved by bioprocessing. During the first 15 min of following time, the control protein isolate decayed drastically from 104 mm to 40 mm, whereas the decay of bioprocessed protein isolates was slower, decreasing to 60–65 mm over 15 min (Figure 7). However, after 30 min following time, all the protein isolate foams were decayed to 30–40 mm of height.

The camera images showed the bubble size distribution of the foams during the 30 min following time (Figure 8). The control foam showed degradation of the bubble structure by coalescence of the bubbles during the first

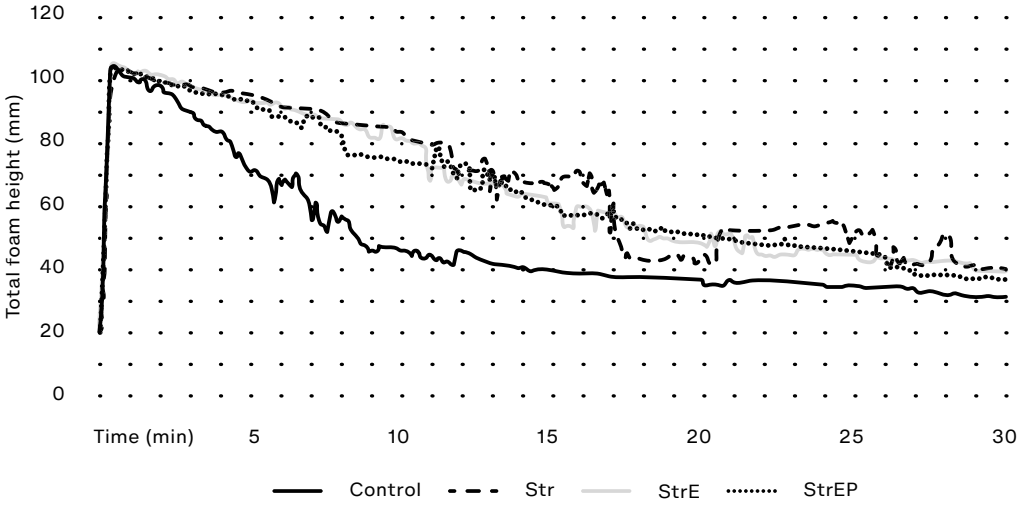

Figure 7. Time-dependent total foam height of the control wheat bran protein isolate and the isolates produced after bioprocessing. In the figure, time zero is the starting point of air conduction to the protein solution. Control, protein isolate produced without bioprocessing; Str, protein isolate bioprocessed with starters (Florapan LA4K); StrE, protein isolate bioprocessed with starters and cell wall-degrading enzymes Bel'ase B210 and Viscoferm; StrEP, protein isolate bioprocessed with starters, cell wall-degrading enzymes and Phytase.

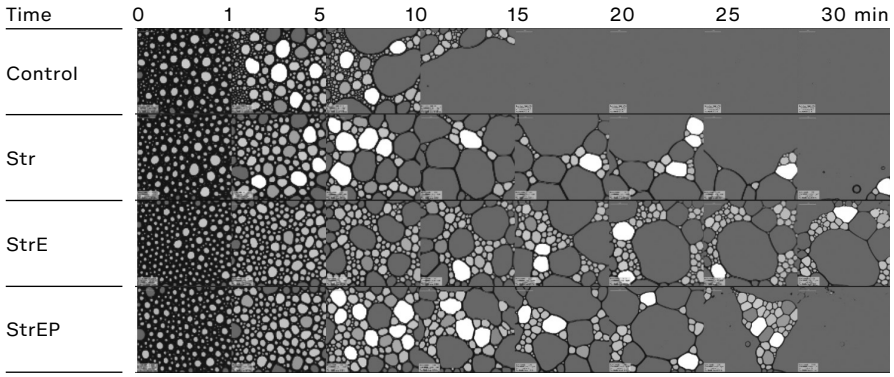

Figure 8. Time-dependent foam bubble structure of the control wheat bran protein isolate and the isolates produced after bioprocessing. In the figure, time zero represents the time point when all air has been conducted to the protein solution. Control, isolate without bioprocessing; Str, isolate bioprocessed with starters (Florapan LA4K); StrE, isolate bioprocessed with starters and cell wall-degrading enzymes Bel'ase B210 and Viscoferm; StrEP, isolate bioprocessed with starters, cell wall-degrading enzymes and Phytase.

10 min of following time, after which the foam height decreased under the camera height and was no longer detectable. The images of the bioprocessed protein isolates also showed coalescence of the bubbles, but with a slower pace, being slowest in the StrE sample.

5.3.3 Bread characteristics

Wheat breads with the addition of bran protein isolates were prepared via the straight-dough method. The bran protein isolates (Control, Str and StrE) were added to breads according to their protein content by substituting 20% of the total energy in the breads with proteins from the isolates to obtain EU Nutrition and Health Claim 'high protein' (Figure 9). For comparison, standard wheat bread was baked without protein isolate addition.

The specific volume was highest in the standard wheat bread (4.3 ± 0.0 ml/g) and was decreased significantly by the addition of the bran protein isolates to 2.4 ± 0.0 ml/g (Control isolate), 2.7 ± 0.2 ml/g (Str isolate) and 2.6 ± 0.0 ml/g (StrE isolate) ($p < 0.05$). No significant difference was found between the breads with added bioprocessed protein isolates ($p > 0.05$).

The textural properties of the breads during storage depended on the added protein isolate (Figure10). The bread with the added control isolate was the hardest of all the breads, and the hardness increased during four days' storage from 3344 g to 5417 g. Wheat bread enriched with bioprocessed protein isolate had significantly lower bread hardness, and the softest bread was obtained by protein isolate bioprocessing with starters, Bel'ase B210 and Viscoferm (StrE isolate). In the bread enriched with StrE isolate, the bread hardness was 1318 g after 1 day of storage and was not changed during the 4 days of storage. Furthermore, after 4 days of baking, the hard-

Figure 9. Wheat bread and wheat breads enriched with wheat bran protein isolates. From left to right: standard wheat bread, control bran protein isolate-, Str protein isolate- (bioprocessed by starters) and StrE protein isolate-enriched breads (bioprocessed by starters and cell wall-degrading enzymes Bel'ase B210 and Viscoferm).

ness was comparable to the standard wheat bread, showing no significant difference between these breads. The bread containing the StrE isolate was noted as sticky and doughy during handling, which was also observed in the instrumental TPA analysis as a significant decrease of springiness and increase of adhesiveness. The springiness of the StrE isolate-containing bread was decreased to 0.7 after 1 day of storage and was not influenced by storage time. A significant adhesiveness (-306 g × sec after one and -263 g × sec after 4 days of storage) was found only in the bread with the added Str isolate. The breads containing the control isolate and Str isolate had significantly lower springiness compared to standard bread. However, no significant differences were found between isolate-enriched breads, having springiness values between 0.89–0.91 after 1 day and 0.89–0.90 after 4 days of storage for both breads.

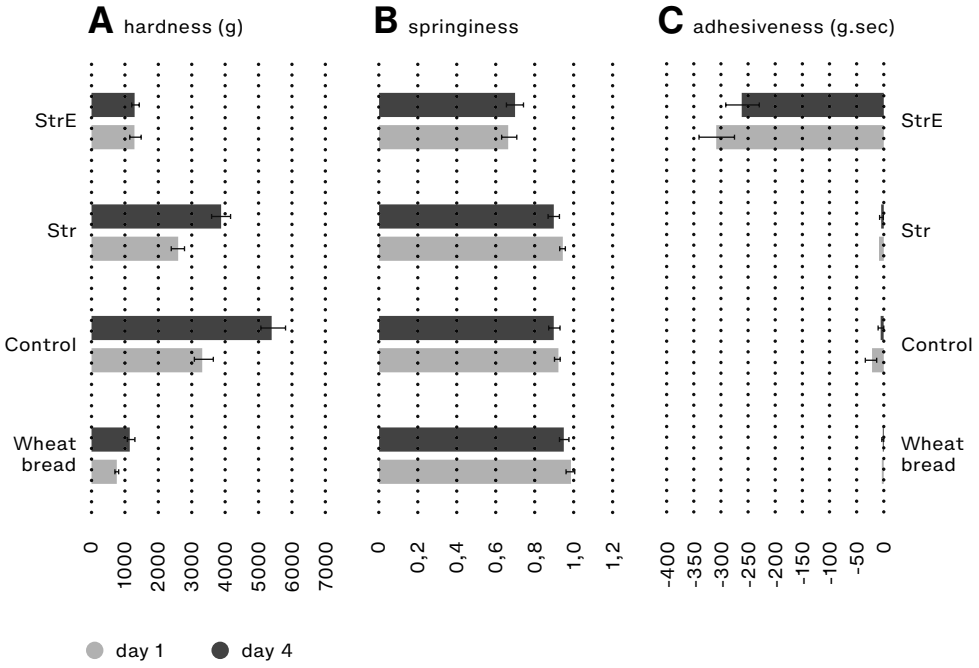

Figure 10. Hardness (A), springiness (B) and adhesiveness (C) of standard wheat bread and wheat breads enriched with wheat bran protein isolates after one and four days after baking. Wheat bread, wheat bread without isolate addition; Control, added control bran protein isolate; Str, added protein isolate bioprocessed by starters; StrE, added protein isolate bioprocessed by starters and cell wall-degrading enzymes Bel'ase B210 and Viscoferm.

6 Discussion

6.1 Improving protein solubilisation by bioprocessing

The first aim of this study was to liberate wheat bran proteins from aleurone cells. The study showed clearly that proteolytic activity during the bran treatments played a crucial role in increasing the protein solubilisation either by exogenous or endogenous enzymes. As shown in study I, the endogenous enzyme activity during bran treatments for 4–16 h was the key factor in increasing the nitrogen solubilisation and protein liberation from the aleurone cells. Acidification and proteolysis during lactic acid fermentation are known phenomena, where the production of lactic and acetic acids by microorganisms decreases the pH, resulting in the activation of endogenous enzymes in bran (Loponen et al. 2004; De Vuyst and Neysens 2005; Katina et al. 2012). The endogenous proteases, especially aspartic proteinase, hydrolyse the proteins, decreasing their molecular size and increasing their water solubility, whereas microbial peptidases hydrolyse the formed peptides further to amino acids (Loponen et al. 2004; Thiele et al. 2004; Gänzle et al. 2008).

Initially, eight commercial carbohydrase-active enzymes without or with very low proteolytic side activities were studied for improved bran protein solubility. The aim was to achieve a fast and significant degradation of bran cell walls that would liberate the proteins within the aleurone cells. However, the carbohydrase-active enzymes did not further improve the nitrogen solubilisation during the 4–16 h of incubation time in comparison to the control bran incubated without enzyme addition. The micrographs confirmed the results, showing the majority of the aleurone cell proteins intact after 6 h of incubation time. Similar outcomes have been shown in previous studies, especially with wheat bran; however, contradicting results of carbohydrases in increasing bran protein solubilisation have been reported as well (Ansharullah et al. 1997; Tang et al. 2003; Heiniö et al. 2012; Jodayree et al. 2012; De Brier et al. 2015). The differing results are likely related to differences in enzyme preparations and treatment conditions. For example, several studies have shown that Celluclast and Econase CE (main activities β-glucanase, endoglucanase and xylanase) do not improve protein solubilisation from cereal brans (Ansharullah et al. 1997; Tang et al. 2003; Heiniö et al. 2012; Jodayree et al. 2012). However, De Brier et al. (2015) obtained a significant increase of solubilised wheat bran proteins, from 22% to 33% and 47%, by treatment (4 h, 40 °C, pH 5) with cellulase or xylanase, respectively. The better performance of the enzyme preparations could be explained by the higher enzyme dosage, ~300 nkat of xylanase/g bran

(in comparison to 100 nkat/g bran used in study I) and the pH adjusted to 5 at the beginning of treatment. The adjusted pH can already activate wheat endogenous enzymes, such as carboxypeptidases, which possibly increased the protein solubilisation in their study (Mikola 1986).

Enzymatic treatments with neutral protease (Corolase 7089) and carbohydrases that contained higher proteolytic side activity (Viscozyme L and Novozym 188) were found to significantly increase nitrogen solubilisation from wheat bran. In previous studies, Viscozyme L (polygalacturonase with protease side activity) and Corolase 7089 (protease) have also been shown to significantly increase the solubilisation of proteins from cereal materials (Ansharullah et al. 1997; Tang et al. 2003; Guan and Yao 2008; Heiniö et al. 2012). In contrast to this study, Heiniö et al. (2012) observed no further improvement of rye flour protein solubilisation by Novozym 188 (β-glucosidase with protease side activity), likely due to the shorter incubation time used (2 h). The effective solubilisation of proteins obtained with Viscozyme L and Novozym 188 could be related to the same phenomena as seen in the micrographs of bran treated with Corolase 7089, in which mainly endosperm proteins were hydrolysed and solubilised. Unfortunately, no studies or microscopic pictures are available from the previous studies or the current study that would reveal the impact of these enzyme preparations on the wheat bran structure, in particular on the aleurone layer.

The pericarp layer in bran contains the majority of the microbial load in wheat including beneficial bacteria and yeasts but also harmful microorganisms (De Vuyst and Neysens 2005; Katina et al. 2012; Sabillón and Bianchini 2016). The long incubation times used in the study (16 h) in uncontrolled conditions could support the growth of harmful microbes and moulds present in bran, and, thus, a long enzyme incubation time with spontaneous fermentation cannot be considered microbiologically safe for industrial processes (Nout 1994; Laca et al. 2006; Zhao et al. 2017). In order to utilise exogenous enzymes in industrial practice, further development and improvement of commercial enzymes should be undertaken to obtain a faster and more efficient solubilisation of bran proteins.

A set of experiments was performed to understand the individual and combined roles of endogenous enzymes, microbial fermentation and cell wall-degrading enzymes Depol 761P (xylanase) and Viscoferm (xylanase and β-glucanase) on the degradation of bran cell walls and the solubilisation of proteins. The solubilisation of bran proteins clearly depended on the incubation time at acidic pH (4.5), optimal for endogenous protease activity. In the treatments with pH adjusted to 4.5 already at the beginning of incubation (End, End/Enz), that is, incubating the bran in acidic conditions for the longest time, the nitrogen solubilisation was the highest (63–75%). Also, controlled fermentation resulted in a high solubilisation of proteins (52%), presumably by the relatively fast acidification. In addition, significant rupture of the bran cell walls and liberation of aleurone proteins within the cell walls were observed in the microstructure of bran fermented in combination with cell wall-degrading enzymes. The rate of acidification and pH decrease during fermentations depends on several factors, such as microorganisms, time, temperature and cereal material used (Thiele et al. 2002; Katina et al. 2004). For example, according to Thiele et al. (2002), sourdough fermentation with LAB starters (*Lactobacillus sanfraciscencis*,

Lactobacillus pontis) decreased the pH to 4.5 within 8 h (30 °C) and achieved a stationary phase after 24 h with a final pH below 4. In the same study, yeast sourdough (*S. cerevisiae* and *C. milleri*) decreased the pH at a significantly slower rate, reaching a higher final pH of 4.9–5.3 within 52 h. A sourdough with both LAB and yeast had a final pH similar to that of LAB sourdough (pH below 4 after 52 h) (Thiele et al. 2002). In spontaneous fermentations, acidification can proceed at a slower rate due to the competition of dominating microorganisms, and a final pH varying from 4.2 (20–25 h, 30–32 °C) to 6.6 (20 h, 20 °C) has been reported (Wehrle and Arendt 1998; Katina et al. 2012).

The results showed clearly that the exogenous enzymes solubilised mainly proteins from endosperm still attached to the aleurone layer and proteins from the aleurone cells broken during milling, whereas the endogenous enzymes liberated and solubilised the proteins within the cells. Indeed, the aleurone layer contains enzymes functioning in grain metabolism and germination (Jerkovic et al. 2010). Presumably, the location of the endogenous enzymes and high substrate specificity towards the grain components were the factors making these enzymes superior in liberating bran proteins within aleurone cells in comparison to the exogenous enzymes.

6.2 Proteolysis during bioprocessing

The degree of proteolysis was significantly influenced by the bioprocessing method. In most cases, the treatments increased the soluble protein, peptide and free amino acid contents but with different intensities, in comparison with the corresponding control sample. Generally, the treatments involving fermentation resulted in more intense protein hydrolysis, evidenced by a lower content of soluble protein and peptides and higher content of free amino acids in comparison to solely enzymatic treatments.

Interestingly, even as the protein solubilisation was highest in bran treatments with endogenous enzymes (End, End/Enz), a modest hydrolysis of proteins was shown by the chemical analysis and electrophoretic patterns of these samples. The moderate hydrolysis of the proteins was likely associated with the lack of microbial activity and slightly higher final pH in these treatments in comparison with the bioprocesses involving fermentation. Furthermore, the endogenous enzymes were effective in solubilising bran proteins rather than causing a significant hydrolysis of them. The enzymatic treatments at pH 6.5 (Enz) or 4.5 (End, End/Enz) activated only the endogenous enzymes optimal for these pHs, and, according to Loponen et al. (2004) the proteolysis in chemically acidified treatments is highly dependent on the pH of the treatment. In the study by Loponen et al. (2004), wheat flour incubated at pH 5.2 caused no detectable protein hydrolysis, whereas treatment in more acidic conditions, at pH 3.7, caused hydrolysis of high molecular weight (HMW) glutenins by the activation of aspartic proteinases. The higher content of peptides and free amino acids in the enzymatic treatment with Depol761P and Viscoferm at a high pH was likely caused by the small protease side activity of the Viscoferm.

The bioprocess treatment with starter fermentation together with the cell wall-degrading enzymes Depol 761P and Viscoferm caused the most intensive protein hydrolysis. Carbohydrases have also been shown in previous studies to intensify the impact of fermentation by providing fermentable sugars faster that promote the microbial growth resulting in faster acidification (Anson et al. 2009; Coda et al. 2014a; 2014b; Hartikainen et al. 2014). Indeed, LAB and yeast consume peptides that are first degraded into amino acids by their intracellular peptidases (Gobbetti et al. 1996). The level of proteolysis and increase of amino acid contents depend on the pH and activity of endogenous enzymes, fermentation time and amino acid consumption by the microorganisms (Thiele et al. 2002; Zotta et al. 2006). In yeast fermentations the content of amino acid increases only after a stationary phase has been achieved (Thiele et al. 2002), whereas in LAB fermentations, the free amino acid content increases or remains constant depending on the LAB species (Thiele et al. 2002; Vermeulen et al. 2005; Zotta et al. 2006). In another study, by Loponen et al. (2007), the free amino nitrogen content increased in wheat sourdough fermented with *L. brevis* but decreased in the same wheat fermentation in which the endogenous enzymes were inactivated by heat treatment. The results indicated that the endogenous enzymes had the primary influential role in sourdough proteolysis. In this study, the pH of chemically acidified samples was 4.5, whereas the pH of bran fermented with starters, Depol 761P and Viscoferm was 3.7 (II). The lower final pH of the fermented bran was closer to the reported pH optimal for aspartic proteinases (3.0–3.5), possibly further enhancing the protein hydrolysis in this treatment (Bleukx and Delcour 2000; Galleshi and Felicioli 1994). Nevertheless, as also shown by Thiele et al. (2002) and by the results obtained in this study, the proteolysis of wheat bran proteins was more intense in the fermented samples, presumably due to both the primary proteolysis caused by endogenous enzymes and the peptidase activity of the microorganisms, resulting in a lower final pH, causing higher aspartic proteinase activity.

6.3 Digestibility of bioprocessed proteins

The nutritional quality of bran proteins caused by bioprocessing was evaluated by determining the protein digestibility with an *in vitro* method. The method gives information about the susceptibility of bran proteins to digestive enzymes and can be used to estimate the digestibility of proteins. The protein digestibility obtained for native bran (15%) was lower than that published in previous studies, where values varying from 29% up to 38% have been reported (Amrein et al. 2003; Coda et al. 2014a). The variation of digestibility values is presumably related to differences in the analytical methods used and/or in the wheat bran, such as alterations in endosperm content and the particle size of the bran. For example, Amrein et al. (2003) used shorter incubation times (pepsin for 30 min, pancreatin 3 h) and a lower pancreatin treatment pH of 7, in comparison to this study and Coda

et al. (2014a), where pepsin hydrolysis was performed for 3 h and pancreatin hydrolysis for 24 h at pH 8. In addition, higher content of endosperm in bran increases the obtained value for protein digestibility, since the endosperm is more susceptible to enzymatic digestion (Amrein et al. 2003; Nordlund et al. 2013). Also, as Coda et al. (2014a) showed, the bran particle size affects the digestibility of proteins. In their study, bran with a particle size of 400 μm had the highest *in vitro* protein digestibility (39%) compared to smaller (29–35%) and larger sized bran (35%).

The digestibility of wheat bran proteins seemed to be related to the hydrolysis degree of the proteins, increasing with more intense proteolysis. Without hydrolysis, as in the solely enzymatic treatments, the bran proteins were more resistant to digestive enzymes. The treatments with only enzymes significantly increased the protein digestibility when compared to control treatments, but the obtained values were significantly lower in comparison with fermented brans. Among the enzymatic treatments, the highest protein *in vitro* digestibility (25%) was obtained in bran treated by the cell wall-degrading enzymes Depol 761P and Viscoferm (Enz). The higher protein digestibility was likely due to the degradation of dietary fibre that was not able to retain water and act as a bulking material, subsequently enabling better access for the digestive proteases to act on the proteins. Although, the protein hydrolysis seemed to have a stronger impact in improving protein digestibility than the degradation of bran cell walls. As shown for legumes, native proteins have a compact and folded structure that is more resistant to proteolysis by digestive enzymes (Kakade 1974; Carbonaro et al. 2012). Also, the pH-shifts during enzymatic digestion (pH 2 in pepsin digestion followed by pH 8 in pancreatin digestion) might cause the aggregation of the proteins, increasing their resistance to digestive proteases.

Bioprocessing involving controlled fermentations clearly improved the *in vitro* protein digestibility and the results are in accordance with previous studies (Harris et al. 2005; Di Lena et al. 1997; Nordlund et al. 2013; Coda et al. 2014a). The improved protein digestibility was associated with more intense proteolysis, which also increased the protein susceptibility towards digestive proteases. Moreover, the addition of hydrolytic enzymes to the fermentation enhanced the degradation of the bran cell walls and further improved the digestibility of the bran proteins. However, the changes in protein digestibility were higher (from 14% to 39%) than reported in the study by Coda et al. (2014a), where the highest increase of wheat bran protein digestibility was 2%, obtained by enzyme-aided fermentation with *L. brevis* E95612, *K. exigua* C81116, xylanase (Depol 740K) and α-amylase (Grindamyl 100). The lower overall impact of bioprocessing on bran protein digestibility could be due to the higher treatment temperature (20°C), resulting in modest acidification (pH decreased from 6.2–6.7 to 5.0–5.6) and subsequent proteolysis, in comparison to this study.

As indicated in the literature review (2.4.2), the presence of phytic acid in the bran aleurone layer may hinder the solubility and enzymatic digestion of proteins by forming insoluble complexes with proteins. In a study by Kies et al. (2006), several feedstuff (e.g. corn, canola meal and rice pollards) formed protein-phytate complexes at pH 2 but not at pHs higher than 4. The pepsin hydrolysis at pH 2 used in their study was able to hydrolyse proteins from insoluble protein-phytate complexes but at a slower rate

compared to soluble protein. In this study, all bran treatments, except that without acidification (Enz), showed a significant increase in phytase activity. However, a straight correlation between phytase activity, bran protein solubility and *in vitro* protein digestibility could not be confirmed.

6.4 Bioprocess-induced changes in bran cell wall integrity

To understand the degradation of bran cell walls during enzymatic and microbial treatments, the contents of WE pentosans and reducing sugars were analysed. The WE pentosans were analysed as they represent hydrolysis products of AX, while reducing sugars represent the overall hydrolysis of carbohydrates in bran. The contents of WE pentosans and reducing sugars were highest after 4 h and 6 h, respectively, after which they were supposedly consumed by bacteria and yeast. The addition of hydrolytic enzymes generally increased or maintained the contents of WE pentosans and reducing sugars throughout the treatments, demonstrating the hydrolysis of bran polysaccharides by these enzymes (I). Based on the carbohydrate analyses, Depol 761P and Viscoferm were the most effective in increasing WE pentosan and reducing sugar contents, respectively, and were thus chosen for following studies for bran cell wall degradation. Although Viscozyme L was found to be effective in increasing the content of reducing sugar, the enzyme preparation was not chosen for further studies due to its relatively high proteolytic side activity.

The enzyme preparation Depol 761P has been shown to effectively degrade wheat and rye cell wall polysaccharides in previous studies as well (Santala et al. 2011; 2013; Petersson et al. 2013; Rosa-Sibakov et al. 2015). In addition, Viscoferm has been used successfully for hydrolysing polysaccharides from extruded wheat bran and pectin from apple pomace (Panfilov et al. 2015; Wikiera et al. 2015). In this study, Depol 761P and Viscoferm were observed to individually hydrolyse endosperm and aleurone cell walls within 6 h. However, a long incubation time (24h) and the combined action of these enzymes were needed for the partial disruption of the aleurone cell walls and the liberation of proteins within the cells. The results are in accordance with Beaugrand (2004), showing that treatment with bacterial xylanase at 60 °C for 24 h caused the disruption of the aleurone cell walls. Indeed, Depol 761P is bacterial xylanase, and they are known to have a higher specificity towards AX with low substitution (Beaugrand et al. 2004; Benamrouche et al. 2002).

The substitution level of AX was presumably the main factor influencing the degradation of the aleurone cell walls. As discussed in the literature review (section 2.1.1), wheat bran contains several histological layers with differing structures and compositions, altering their susceptibility towards enzymatic degradation. The cell walls in the aleurone layer and testa are constructed of AX with lower substitution (A/X ratio 0.1–0.5) in comparison to pericarp (A/X ratio ~1.2) (Barron et al. 2007). Despite

the endosperm cell walls having differences in structure and composition compared to the aleurone layer (e.g. thinner cell walls, lower β-glucan content and AX ratio 0.5–0.8), they are structurally closely related to each other, explaining their effective hydrolysis during xylanase treatment as well (Saulnier et al. 2012). Even though Depol 761P and Viscoferm were active against the aleurone cell walls, the enzymes were not effective enough for complete aleurone cell wall degradation, demonstrating the complex nature of bran cell walls and the requirement for endogenous enzyme activity for significant protein solubilisation.

Bran treatment activating only endogenous enzymes (End) significantly increased the reducing sugar content, suggesting that enzymes such as β-amylases and β-glucosidases were more active than pentosanases (Gänzle 2014; Chaquilla-Quilca et al. 2018). The combined action of endogenous enzymes and the addition of cell wall-degrading enzymes increased the release of WE pentosans and reducing sugars, showing synergistic action between the enzymes on hydrolysing bran polysaccharides. The bioprocessing method involving starter fermentation and cell wall-degrading enzymes resulted in the most intense breakdown of bran cell walls, as shown by the micrographs. Thus, the synergistic action of endogenous, exogenous and microbial enzymes was needed for the extensive degradation of bran aleurone cell walls. Similar conclusions have been made with the bioprocessing of rye and wheat bran by fermentation with LAB and baker's yeasts in combination with starch and cell wall-degrading enzymes (Katina et al. 2012; Nordlund et al. 2013; Coda et al. 2014a).

The low ability of the several studied commercial enzyme preparations to cause substantial degradation of bran aleurone cell walls within a short time (4–6 h) was likely related to (1) the complex and insoluble nature of the bran cell walls, (2) the structural compounds within the wall polysaccharides and (3) the presence of enzyme inhibitors.

As reported also by other authors, the pericarp layer was found to be resistant to enzymatic and microbial degradation, thus physically preventing the liberation of proteins within aleurone cells from the pericarp side of bran (Beaugrand et al. 2004; Benamrouche et al. 2002). Indeed, the pericarp layer is constructed of insoluble fibre, highly branched AX and cellulose reinforced with lignin and phenolic acids (Hemery et al. 2007). In more detail, the resistant nature of the pericarp layer towards enzymatic degradation (in addition to its highly branched AX) has been suggested to be due to lignin and dehydrodiferulates acting in AX cross-linking, making the layer rigid, physically strong and reluctant to hydrolysis by xylanases (Antoine et al. 2003; Beaugrand et al. 2004; Parker et al. 2005). In addition to the structural differences within bran layers, wheat bran has been shown to be more resistant to enzymatic degradation than rye bran. Petersson et al. (2013) reported that rye bran dietary fibre was more susceptible to solubilisation by microbial xylanases, probably due to the lower substitution level of rye AX (0.40) in comparison to wheat AX (0.45). The reported differences in the A/X ratio between the rye and wheat AX were low, however, the higher hydrolysis of rye bran could be explained by the differences in the bran polysaccharide composition: rye bran contains two times more β-glucan (4.2–5.3 g/100 g) and fructan (6.6–7.2 g/100 g) and approximately half the amount of cellulose (5.0–6.5 g/mol) in comparison with wheat bran (Kamal-Eldin et al. 2009).

As study I showed, the aleurone cell walls were resistant to enzymatic hydrolysis by the commercial enzymes within short incubation times (4–6 h) probably due to the structural compounds within the cell walls. According to Benamrouche et al. (2002), ferulic acid within the aleurone polysaccharides does not limit the degradation of AX, whereas other authors have suggested that protein-ferulic acid cross-links partly resist the degradation of AX (Rhodes and Stone 2002; Klepacka and Fornal 2006; Stone 2006). These cross-links formed by ferulic acid and proteins could inhibit the action of the enzymes by blocking or burying the possible active sites for enzymes. Viscozyme L and Depol 740L have been reported to contain ferulic acid esterase as side activity but no indications for improved cell wall breakdown were detected with these enzymes in this study, suggesting that ferulic acid had a minor role in hindering cell wall degradation (Moore et al. 2006; Anson et al. 2009). Furthermore, the enzyme preparations with proteolytic side activities (Novozym 188, Viscozyme L), which in theory could hydrolyse the protein-constructed cross-links, were effective in solubilising the bran proteins, but the ability to improve especially the degradation of aleurone cell walls could not be confirmed from the results.

The third factor hindering aleurone cell wall degradation could be the xylanase inhibitors present in wheat bran. At least three types of inhibitors have been identified from the wheat bran intermediate layer, including the *Triticum aestivum* xylanase inhibitor (TAXI), xylanase-inhibiting proteins (XIP) and thaumatin-like proteins (TLXI) (Jerkovic et al. 2010). All these enzyme inhibitors are active against microbial and/or fungal xylanases, thus also inhibiting the commercial enzyme preparations (Gebruers et al. 2001; Gys et al. 2004; Jerkovic et al. 2010). As reviewed by Berrin and Juge (2008), xylanases from *Aspergillus niger*, *Trichoderma longibrachiatum* and *Bacillus* spp. have sensitivity towards at least one of the xylanase inhibitors in wheat. Most of the enzymes used in this study – Depol 761P, Bel'ase B210, Glucanase 5 XL, Viscoferm, Novozym 188, Corolase 7089 and Phytase – were produced by these microorganisms, thus likely having sensitivities towards wheat xylanase inhibitors.

6.5 Technofunctionality of bioprocessed bran protein isolates

In addition to improved bioavailability and nutritional quality, the technofunctional properties of bran proteins address their usefulness in food applications. In the current study, the bioprocess-induced changes in technological properties, such as protein solubility, foaming and the emulsifying properties of wheat bran proteins, were established. In addition, changes in bread texture were examined from wheat breads enriched by bran protein isolates.

The bioprocessing of bran had an impact on the chemical composition of bran protein isolates, which in turn altered the technofunctional properties

of bran proteins. The major observation was that bioprocessing increased the protein content in isolates from 67% up to 82% due to the hydrolysis of starch and soluble fibre. Additional proteins were identified from the bran protein isolate bioprocessed by fermentation with the addition of Bel'ase B210, Viscoferm and Phytase. The identified globulins, chitinase, β-amylase and LMW glutenins indicated the liberation and improved solubilisation of proteins from the aleurone layer and remnants of endosperm. The protein yield was not influenced by bioprocessing, being 44–48% in all protein isolates. The obtained protein yields are significantly higher than reported yields for wheat germ (18–28%) or rice bran (34–36%) made by wet-alkaline extraction and isoelectric precipitation. In a study by Wang et al. (1999), the xylanase and phytase pre-treatment of rice bran resulted in a significantly higher protein yield in bran protein isolates (up to 75%). However in this study, bioprocessing with the addition of Phytase (StrEP) was not shown to improve the protein yield of bran protein isolates.

The protein solubility obtained for the control wheat bran protein isolate was in accordance with Idris et al. (2003). A typical protein solubility for plant proteins is a U-shaped curve according to solution pH, and a wide range of protein solubility values (5–78%) at pH 4–8 is found in the literature on protein isolates and concentrates from cereal brans (Wang et al. 1999; Tang et al. 2003; Guan et al. 2007; Prosekov et al. 2018). The low protein solubility of the isolates at a pH range of 4–7 was expected in this study since the precipitation of proteins was performed at a pH within this area (pH 5.5). The protein solubility was likely also influenced by the lyophilisation of the isolates as it is known to denaturate proteins by removing the bound water (Crowe et al. 1990). Interestingly, the bioprocessed bran protein isolates had lower protein solubility than the control protein isolate throughout pH 4–7, which could be linked to the more heterogeneous protein composition in the isolates. As discussed in the literature review (sections 2.2/2.3/2.5.2), the bran proteins are composed of a range of different proteins with varying pIs from 3–10 (Laubin et al. 2008; Gao et al. 2009). The modest hydrolysis and improved solubilisation of bran proteins by bioprocessing increased the extractability and precipitation of additional proteins that were absent from the control isolate. Also, the higher content of starch and soluble fibre in the control isolate may have a role in improving the protein solubility. Indeed, protein-polysaccharide complexes with certain anionic carbohydrates can enhance the solubility of protein isolates, as shown in the canola protein isolate (Klassen et al. 2011).

The bioprocessed bran protein isolates had a stronger net charge and lower surface hydrophobicity, but the changes were not enough to increase the surface activity of proteins to form stable oil-in-water emulsions. However, the foaming stability was increased two-fold by bioprocessing with fermentation and cell wall-degrading enzymes. Previous studies have shown quite unanimously that the foaming stability of cereal brans, oilseed and legume protein isolates decreases according to an increased hydrolysis rate (Vioque et al. 2000; Guan et al. 2007; Meinlschmidt et al. 2016b; Prosekov et al. 2018). The contradicting results suggest that rather than bioprocessing causing significant hydrolysis of bran proteins, it improved the solubilisation of additional bran proteins, such as the globulins and HMW glutenins identified from StrEP isolate bioprocessed with starters, cell wall-degrading

enzymes (Bel'ase B210 and Viscoferm) and Phytase. These proteins, in turn, had more sufficient technological properties, such as flexibility, that improved their adjustment at the air-water interface, resulting in a more stable foam structure (Damodaran 2008).

The improved foaming properties were reflected in breads enriched with bioprocessed protein isolates. Wheat bread can be considered a baked foam, where initially air is incorporated into a liquid starch-gluten network. The stability to retain air in the foam depends on the viscoelasticity of the starch-gluten network and the surface-active components (e.g. proteins, soluble fibre, lipids) within the air-water interface (Mills et al. 2003). Hence, wheat bread was a logical food application for studying the applicability of bioprocessed protein isolates in a food product.

The high water adsorption of bread ingredients can lead to lower dough volume and the increased hardness of bread. The addition of wheat bran protein isolates decreased the loaf volume and was not influenced by bioprocessing, indicating that the bioprocess-induced changes in the isolates had little impact on the volume of breads. Also in previous studies, the addition of cereal or oilseed protein isolates has been reported to decrease loaf volume and unanimously suggested to be caused by gluten dilution and the mechanical disruption of the gluten network by the isolate particles (Jiamyangyuen et al. 2005; Paraskevopoulou et al. 2010; Chinma et al. 2015).

The bread enriched with isolate bioprocessed by starters, Bel'ase and Viscoferm was softest and had significantly adhesive crumb and low springiness. Sticky bread crumb is typical for breads with high xylanase activity, caused by the excessive amount of hydrolysed AX, which reduces the water-holding capacity of the dough (McCleary 1986; Courtin and Delcour 2002). Thus, the added enzymes in the bran bioprocessing were also present in the protein isolates and activated during baking, resulting in sticky bread crumb and loss of crumb springiness.

Between the control protein isolate and protein isolate made after LAB and yeast fermentation, the differences in the protein fractions of the isolates presumably had the most significant impact in bread hardness. According to previous literature, wheat bread enriched with legume-, seed- or cereal bran-based concentrates or isolates resulted in increased bread hardness in comparison to standard wheat bread (El-Adawy 1997; Jiamyangyuen et al. 2005; Paraskevopoulou et al. 2010; Chinma et al. 2015). Paraskevopoulos et al. (2010) suggested that the higher firmness of lupine protein isolate-enriched wheat bread was due to the thickening and strengthening of the crumb air cell walls by the lupine protein particles. Nevertheless, Chinma et al. (2015) showed that wheat bread enriched with 10% rice bran protein concentrate made after yeast fermentation decreased the bread hardness by 11% in comparison to bread enriched with rice bran protein concentrate prepared after spontaneous fermentation. The more intense decrease of bread hardness could be linked to the faster acidification and more intense bran modifications by the yeast in comparison to the spontaneous fermentation. Paraskevopoulos et al. (2010) reported that albumin-rich lupine protein isolates delayed bread firming during 24 h storage, whereas globulin-rich lupine protein isolate had no effect on bread staling. After 48 h, breads with either type of protein isolates had lower bread hardness in comparison to wheat bread. The results indicate that endogenous enzymes

were active during the baking, thus improving the bread texture during storage. Indeed, at low concentrations, amylases, xylanases and proteases have been reported to delay the staling of wheat bread (Mathewson 2000; Haros et al. 2002; Hug-Iten et al. 2003). The enzymatic activities in the fermented protein isolate, such as β-amylase, at least partly explain the softer bread crumb and delayed staling of the breads.

In addition to the protein profiles of the isolates, another factor influencing bread hardness was the presence of starch and soluble fibre. The control isolate had the highest contents of starch and WE pentosans as well as the highest bread hardness during storage. Starch retrogradation is the initial cause of increased bread firmness, thus less starch able to retrogradate could at least in part explain the delayed staling of breads with fermented isolate (Biliaderis et al. 1995). Also, HMW AX are known to absorb water in dough, thus either slowing or intensifying bread staling depending on the amount of available water in the system (Courtin and Delcour 2002). In the control protein isolate, the content of WE pentosans was rather low (2.5%) and decreased by bioprocessing to 1.1–1.5%. In contrast to this study, Biliaderis et al. (1995) obtained softer bread crumb despite the increased rate of starch retrogradation with the addition of water-soluble AX (0.5–1.3%) to wheat dough in similar amounts as added in this study by the control isolate.

The practical study on applying bioprocessed bran proteins to wheat bread was relevant to see the interactions of the protein isolates with other components in food matrices since these are often not observed in more simple chemical analyses and technological tests. More research is still needed to fully understand the mechanism of bran proteins on bread structure formation.

6.6 Limitations of the study

The present study aimed to examine bioprocessing as a tool to improve wheat bran protein bioavailability, nutritional quality and technological properties for food applications. Wheat bran is a challenging raw material for research due to its uneven composition. As mentioned in the literature review (section 2.1.1), side streams are collected in mills without standardisation of the composition, meaning that, for example, the contents of endosperm and germ and the microbial load can vary between brans (Elliott et al. 2002). Also in this study, wheat brans were obtained from different mills and had variations in chemical composition, for example, in fibre and protein content. Nevertheless, a promising observation was that the bioprocessing resulted in remarkably similar outcomes regardless of the wheat bran batch, indicating that bioprocessing as a method was not sensitive to variations in the raw material. Still, in order to promote the utilisation of side streams as main streams in food production, the variation in bran composition should be controlled for food production aiming at consistent end-product quality.

Compromises had to be made in the designing of the experiments in order to either improve the research value or to maintain the applicability for industrial processes. For example, commercial enzyme mixtures were chosen over purified enzymes due to their easier availability for industrial applications. The most crucial enzyme activities or the required synergistic actions of certain enzyme activities for cell wall degradation and protein solubilisation could not be distinguished from the enzyme mixtures with varying enzyme side activities. Similarly, even though the specific LAB and yeast species from the VTT Culture Collection showed good fermentation performance on cereal brans, they were substituted by commercial starter cultures as the latter are readily available for industry.

Some improvements were also made during the studies. Different particle-sized brans, as well as varying bran-water ratios, were used in the bran treatments. In the first study, milled wheat bran was chosen for its mechanical disruption of bran cell walls prior to enzymatic hydrolysis, which already enhances protein liberation. The high water content in the treatments (1:10 w/v) was used to ensure enzymatic performance. In the second study, coarse bran was chosen to decrease the processing steps and improve the applicability of bioprocessed brans at the industrial scale. However, in the pre-experiments of study III, the milling of bran was noted to significantly improve the protein solubilisation, thus milled bran was used for the experiments. However, the bran-water ratio had to be adjusted according to the bran particle size. With a bran-water ratio of 30:70 w/v, coarse bran absorbed almost all the water, and low amounts of the soluble fraction could be collected. Thus, higher (20:80 w/v) water content in coarse bran treatments was needed to obtain adequate amounts of soluble fraction for analysis. With milled bran the water adsorption was lower, thus 30:70 (w/v) was optimal for protein solubilisation and to collect the soluble fraction for analysis.

The wheat bran protein isolates in this study were prepared by wet-alkaline extraction and isoelectric precipitation. Wet-alkaline extractions of plant proteins followed by isoelectric precipitation is currently used in the industry in contrast to other methods still in the experimental phases, such as ultrafiltration (Alonso-Miravalles et al. 2019). The high extraction pH used in the study may negatively affect the functional properties of bran proteins causing the denaturation, cross-linking and racemisation of amino acids (Sozer et al. 2017). Another limitation in this study was that isoelectric precipitations precipitates proteins with the selected pH, resulting in a rather low protein yield. By sequential protein precipitation with selected precipitation pH, more proteins could be obtained in the final isolate from the bran material. In addition, the production of protein isolates requires rather high amounts of water and is performed in harsh conditions (pH around 11). Further optimisation of the isolation method, such as extraction pH, sequential protein precipitation and dry matter-to-solution ratio, would be interesting to study in order to improve the protein isolation yield with milder treatment conditions without compromising the technological properties. Thus, in order to use the side stream proteins in large scale production, more economically feasible and sustainable protein isolation technologies should be developed.

The solubilisation of bran proteins after different bioprocessing methods was evaluated by analysing the total solubilised nitrogen content by the

Kjeldahl and Dumas combustion methods. These methods are based on nitrogen liberation at high temperatures and were the most reliable analytical methods to evaluate the overall nitrogen solubilisation from bran. In addition, these methods are the official standard methods of AOAC International and AACC International (AACC, 2003; AOAC 2019). However, the Kjeldahl and Dumas methods lack selectivity towards protein-based nitrogen simultaneously measuring nitrogen from other compounds as well (e.g. B vitamins), which could result in an over-estimation of protein content (Moore et al. 2010). Furthermore, the conversion factor used may influence the accuracy of the calculated protein content and considerable debate on the subject has taken place, as reviewed by Moore et al. (2010) and Mariotti et al. (2008). Nevertheless, in study III, a conversion factor of 6.31 from nitrogen to protein content was used, as recommended for wheat bran by the FAO (FAO 2002). The protein content calculated with the recommended conversion factor was observed to be in line with the total amino acid content analysed from hydrolysed proteins by ultra-high performance liquid chromatography. Another protein analysis method used was the Bradford method, which cannot detect peptides or proteins with an MW under 3,000 g/mol. Thus, the Bradford method was used for the estimation of soluble protein content but not for the overall content of proteins and protein derivatives. For a more comprehensive evaluation of protein hydrolysis after bioprocessing methods, several analysis methods for protein, peptide and amino acid contents were performed (II).

Another limiting factor in the analytical methods used was the determination of WE pentosan and reducing sugar contents to represent the hydrolysis of bran cell walls. These methods could be used to show the hydrolysis of bran polysaccharides in short incubation times (< 6 h). However, with longer incubation times (> 6 h) the microorganisms consumed the carbohydrates and the obtained results were not reflecting the bran polysaccharide degradation. Two bioprocessing treatments (carbohydrases and LAB fermentation with added carbohydrases) were analysed additionally by microscopy to give more detailed information on the bioprocess-induced changes in bran structure during fermentation.

The wheat bran proteins within aleurone cells are stored as globoids composed of proteins and phytic acid (Bohn et al. 2007; Bechtel et al. 2009; Regvar et al. 2011). As indicated in the literature review (section 2.4.2), phytic acid can form insoluble complexes with proteins, thus decreasing their solubility. In study III, phytase was added to the bioprocess to examine whether the degradation of phytic acid would further enhance the solubilisation and extractability of bran proteins. Based on the pre-experiments, bioprocessing with fermentation, cell wall-degrading enzymes and Phytase significantly decreased the phytic acid content in bran in comparison to the same bioprocess without the Phytase addition (data not shown). Despite this, no significant improvements in protein yield were obtained by the addition of Phytase, and only minor changes were observed in the biochemical and technological properties of the isolate. The results encourage further studies to understand the interactions between bran proteins and phytic acid.

6.7 Future prospects

This study showed that bioprocessing is an effective and feasible tool to improve wheat bran protein solubility while simultaneously enhancing their digestibility and technological properties. With an estimated 120 M tons of wheat side streams produced annually and an average of 18% of protein content in bran, it can be roughly calculated that 21.6 M tons of bran protein is produced yearly. According to the World Resources Institute, global protein consumption is 86 grams/day/person (Ranganathan et al. 2016). With bioprocessing involving lactic acid fermentation, 51–52% of the proteins were solubilised, thus, in theory, fulfilling the daily protein consumption for 360 million people. The estimations show the massive scale of a potential plant-based protein source from wheat bran.

However, more research is needed to optimise the bioprocessing conditions for targeted end-products. Applying bioprocessed wheat bran as such to solid and semi-solid foods would be economically the most feasible way to utilise the bran. Indeed, the fermentation of wheat bran has been shown to improve the technological properties of bread and extruded cereal products (Dunaevsky et al. 1989; Salmenkallio-Marttila et al. 2001; Katina et al. 2012; Hartikainen et al. 2014). A challenge in using bioprocessed wheat bran is the low final pH, which prevents high substitution levels in foods if consumer acceptance is taken into account. Another challenge is the application of high amounts of fibre in baking products since they generally have detrimental effects on the textural properties (Hemdane et al. 2016). Thus, other types of cereal-based products, such as crackers and crispbreads, would be interesting to study, aiming at improving protein quality and digestibility together with modified bran fibre fraction. The use of bran proteins without the challenges associated with fibre fraction would be possible by isolating the water-soluble fraction as a protein concentrate. With this approach, a challenge – or advantage – would be the concomitant compounds present in the soluble fraction, such as phenolic acids, carbohydrates, sugars and minerals. In this study, protein concentrates were not examined due to the interference of co-passing compounds in understanding the role of bioprocessing on the biochemical and technological properties of bran proteins. However, the interactions of proteins with other compounds present in the soluble fraction would be interesting to study in future.

As shown in this study, high protein solubilisation with long bioprocessing times caused the simultaneous hydrolysis of proteins as well. The higher protein hydrolysis rate was associated with improved protein digestibility. The intensive protein hydrolysis could be exploited in producing bioactive peptides, such as ACE inhibitory peptides, for health-promoting products (Rizzello et al. 2008; Coda et al. 2012).

Bioprocessing was also shown to produce protein isolates with high protein content and low soluble fibre content. Thus, bioprocessing has great potential to be used as a clean-label pre-treatment for cereal-based materials in the production of protein isolates. The improved foaming stability obtained in bioprocessed protein isolates could be used, in addition to bread, in other types of foam-structured foods, such as extruded snacks or even plant-based ice creams. However, a substantial amount of work still needs

to be done to apply bioprocessed protein isolates to food products. For example, a suitable defatting method, such as supercritical carbon dioxide extraction, the inactivation of excess enzymatic activity and the optimisation of the baking process, needs to be applied to improve the storage and textural properties of isolate-enriched breads. Furthermore, the taste, flavour and colour of protein isolates were likely influenced by bioprocessing, and the impact of different bioprocessing methods (i.e. fermentation and addition of enzymes) on the sensory profile of isolate-enriched breads would be interesting to define. Finally, as the work aimed to improve the bioavailability and nutritional quality of bran proteins, *in vivo* trials with humans is of interest to determine the full potential of wheat bran proteins in food. *In vivo* studies especially with wheat bran proteins have not been yet performed.

7 Conclusions

In the present work, different combinations of hydrolytic enzymes and microbial fermentation were used to increase the liberation and solubilisation of bran proteins with simultaneous improvement of nutritional quality and technological functionality.

Treatments by enzymes with only carbohydrase activities were effective in the solubilisation of bran cell walls on the aleurone side of bran (i.e. endosperm and aleurone cell walls) but were not able to liberate and solubilise the bran proteins from aleurone cells. Enzyme preparations with proteolytic main or side activities were effective in increasing the solubilisation of bran proteins, showing that the proteolytic activity was required for improved protein solubilisation. Even with the most effective enzyme preparation, Corolase 7089 (58% protein solubilised in 4 h), the enzyme was effective in solubilising and hydrolysing mainly endosperm proteins, whereas the proteins within aleurone cells remained intact. The first study showed that proteolysis by endogenous proteases is needed in microbially controlled conditions in order to liberate the proteins within the wheat aleurone cells.

The individual and combined actions of enzymatic treatments (exogenous cell wall-degrading carbohydrases and endogenous enzymes) and microbial fermentations were studied to examine their role in modifying the wheat bran and the bioavailability and nutritional quality of its proteins. The results showed that the solubilisation of bran proteins was dependent on the incubation time in acidic conditions optimal for endogenous proteases, whereas the hydrolysis rate and nutritional quality of the proteins were related to the microbial activity during the treatment. The activation of endogenous enzymes in bran by chemical acidification most significantly increased the protein solubilisation, up to 75%, causing modest proteolysis and the simultaneous improvement of *in vitro* protein digestibility (from 14% to 20%). Lactic acid fermentation by LAB and yeast in combination with cell wall-degrading enzymes (Depol 761P and Viscoferm) was found to be the most beneficial and microbially safe method for the gradual acidification and subsequent activation of endogenous proteolytic enzymes in bran. This bioprocessing method resulted in significant protein solubilisation (52%), and the hydrolysed proteins during the treatment resulted in significantly improved *in vitro* protein digestibility (39%). By a rough estimation of the current wheat production rates, 11.3 M tons of protein/year could be utilised as good-quality protein for human nutrition instead of using them mainly as feed, as currently is the case. On the basis of this study, bioprocessing has significant potential to improve the solubility as well as the nutritional quality of bran proteins with a feasible and ‘clean-label’ method.

This study was the first to show bioprocessing as an effective tool to modify the chemical composition and to improve the technological properties of bran protein isolates. Bioprocessing with lactic acid fermentation with or without the addition of cell wall-degrading enzymes and Phytase increased the protein content of protein isolates up to 80% by degrading WE pentosans and starch. In general, the bioprocessing of wheat bran liberated and solubilised additional proteins to the isolates that were suggested to induce the physicochemical modifications. The more heterogeneous protein profile in the bioprocessed protein isolates likely reduced the solubility in comparison to the control isolate with a more homogeneous protein profile. Even though the emulsifying properties were not affected by bioprocessing, the foaming stability was almost doubled by bioprocessing with lactic acid fermentation and enzymes. Throughout the study, bioprocessing with the addition of enzymes had the most intensive effect in regard to modifications in the chemical composition of bran, the nutritional quality of proteins and the technofunctional properties of the protein isolates. However, the fermentation of wheat bran by LAB and yeast without the enzyme addition was found to be most optimal for bread baking in terms of bread texture. The bran protein isolates were added to wheat bread according to their protein contents, substituting 20% of the total energy by proteins from the isolates. The breads containing protein isolate fermented by LAB and yeast delayed staling and showed lower firming during 4 days of storage in comparison to bread enriched with the control protein isolate made without bioprocessing. With the optimisation of the baking process, significant improvements in the textural properties of the bread could be achieved, and the results of this study show that the applicability of bran protein isolates to food products is highly promising.

References

[AACC] American Association of Cereal Chemists. 2003. Approved Methods of Analysis.11th Ed. St. Paul (MN): AACC International.

Akeson WR, Stahmann MA. 1964. A pepsin pancreatin digest index of protein quality evaluation. J Nutr 83:257–261.

Alonso-Miravalles L, Jeske S, Bez J, Detzel A, Busch M, Krueger M, Wriessnegger CL, O'Mahony CL, James A, Zannini E, Arendt EK. 2019. Membrane filtration and isoelectric precipitation technological approaches for the preparation of novel functional and sustainable protein isolate from lentils. Eur Food Res Technol EFRT-19-00:EFRT-19-0094.R1.

Amrein TM, Gränicher P, Arrigoni E, Amadò R. 2003. *In vitro* digestibility and colonic fermentability of aleurone isolated from wheat bran. LWT - Food Sci Technol 36:451–460.

Ansharullah, Hourigan JA, Chesterman CF. 1997. Application of carbohydrases in extracting protein from rice bran. J Sci Food Agric 74:141–146.

Anson NM, Selinheimo E, Havenaar R, Aura A-M, Mattila I, Lehtinen P, Bast A, Poutanen K, Haenen GRMM. 2009. Bioprocessing of wheat bran improves *in vitro* bioaccessibility and colonic metabolism of phenolic compounds. J Agric Food Chem 57:6148–6155.

Antoine C, Peyron S, Lullien-Pellerin V, Abecassis J, Rouau X. 2004. Wheat bran tissue fractionation using biochemical markers. J Cereal Sci 39:387–393.

Antoine C, Peyron S, Mabille F, Lapierre C, Bouchet B, Abecassis J, Rouau X. 2003. Individual contribution of grain outer layers and their cell wall structure to the mechanical properties of wheat bran. J Agric Food Chem 51:2026–2033.

Bailey MJ, Biely P, Poutanen K. 1992. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257–270.

Bailey MJ, Nevalainen KMH. 1981. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. Enzyme Microb Technol 3:153–157.

Bailey MJ, Pessa E. 1990. Strain and process for production of polygalacturonase. Enzyme Microb Technol 12:266–271.

Barron C, Samson MF, Lullien-Pellerin V, Rouau X. 2011. Wheat grain tissue proportions in milling fractions using biochemical marker measurements: Application to different wheat cultivars. J Cereal Sci 53:306–311.

Barron C, Surget A, Rouau X. 2007. Relative amounts of tissues in mature wheat (*Triticum aestivum* L.) grain and their carbohydrate and phenolic acid composition. J Cereal Sci 45:88–96.

Beaugrand J, Reis D, Guillon F, Debeire P, Chabbert B. 2004. Xylanase-mediated hydrolysis of wheat bran: evidence for subcellular heterogeneity of cell walls. Int J Plant Sci 165:553–563.

Bechtel DB, Abecassis J, Shewry PR, Evers AD. 2009. Development, structure, and mechanical properties of the wheat grain. In: Khan K, Shewry PR, editors. Wheat chemistry and technology. 4th Ed. St. Paul (MN): AACC International, Inc. p. 51–95.

Benamrouche S, Crônier D, Debeire P, Chabbert B. 2002. A chemical and histological study on the effect of (1→4)-β-endo-xylanase treatment on wheat bran. J Cereal Sci 36:253–260.

Bernfeld P. 1955. Amylase α and β. In: Colowick SP, Kaplan NO, editors. Methdos in Enzymology New York (NY): Academic Press. p. 149–158.

Berrin J-G, Juge N. 2008. Factors affecting xylanase functionality in the degradation of arabinoxylans. Biotechnol Lett 30:1139–1150.

Biliaderis CG, Izydorczyk MS, Rattan O. 1995. Effect of arabinoxylans on bread-making quality of wheat flours. Food Chem 53:165–171.

Bleukx W, Brijs K, Torrekens S, Van Leuven F, Delcour JA. 1998. Specificity of a wheat gluten aspartic proteinase. Biochim Biophys Acta - Protein Struct Mol Enzymol 1387:317–324.

Bleukx W, Delcour JA. 2000. A second aspartic proteinase associated with wheat gluten. J Cereal Sci 32:31–42.

Bohn L, Josefsen L, Meyer AS, Rasmussen SK. 2007. Quantitative analysis of phytate globoids isolated from wheat bran and characterization of their sequential dephosphorylation by wheat phytase. J Agric Food Chem 55:7547–7552.

Bohn L, Meyer AS, Rasmussen SK. 2008. Phytate: impact on environment and human nutrition. A challenge for molecular breeding. J Zhejiang Univ Sci B 9:165–191.

Brandolini A, Hidalgo A. 2012. Wheat germ: not only a by-product. Int J Food Sci Nutr 63:71–74.

Breddam K, Sorensen B, Svendsen I. 1987. Primary structure and enzymatic properties of carboxypeptidase II from wheat bran. Carlsberg Res Commun 52:297–311.

Brijs K, Courtin CM, Goesaert H, Gebruers K, Delcour JA, Shewry PR, Henry RJ, Nicolas J, Potus J, Garcia R, Davidou S. 2009. Enzymes and enzyme inhibitors endogenous to wheat. In: Khan K, Shewry PR, editors. Wheat chemistry and technology. 4th Ed. St. Paul (MN): AACC International, Inc. p. 401–435.

Brouns F, Hemery Y, Price R, Anson NM. 2012. Wheat aleurone: separation, composition, health aspects, and potential food use. Crit Rev Food Sci Nutr 52:553–568.

Buri RC, Reding W, Gavin MH. 2004. Description and characterization of wheat aleurone. Cereal Foods World 49:274–282.

Bye JW, Cowieson NP, Cowieson AJ, Selle PH, Falconer RJ. 2013. Dual effects of sodium phytate on the structural stability and solubility of proteins. J Agric Food Chem 61:290–295.

Carbonaro M, Maselli P, Nucara A. 2012. Relationship between digestibility and secondary structure of raw and thermally treated legume proteins: a Fourier transform infrared (FT-IR) spectroscopic study. Amino Acids 43:911–921.

Chaquilla-Quilca G, Balandrán-Quintana RR, Huerta-Ocampo JÁ, Ramos-Clamont Montfort G, Luna-Valdez JG. 2018. Identification of proteins contained in aqueous extracts of wheat bran through a proteomic approach. J Cereal Sci 80:31–36.

Cheryan M. 1980. Phytic acid interactions in food systems. Crit Rev Food Sci Nutr 13:297–335.

Chinma CE, Ilowefah M, Shammugasamy B, Mohammed M, Muhammad K. 2015. Effect of addition of protein concentrates from natural and yeast fermented rice bran on the rheological and technological properties of wheat bread. Int J Food Sci Technol 50:290–297.

Chinma CE, Ilowefah M, Shammugasamy B, Ramakrishnan Y, Muhammad K. 2014. Chemical, antioxidant, functional and thermal properties of rice bran proteins after yeast and natural fermentations. Int J Food Sci Technol 49:2204–2213.

Chung OK, Ohm J-B, Ram MS, Howitt CA. 2009. Wheat Lipids. In: Khan K, Shewry PR, editors. Wheat. Chemistry and Technology. 4th Ed. St. Paul (MN): AACC International, Inc. p. 363–399.

Cleemput G, Hessing M, Van Oort M, Deconynck M, Delcour JA. 1997. Purification and characterization of β-D-xylosidase and an endo-xylanase from wheat flour. Plant Physiol 11:377–386.

Coda R, Rizzello CG, Pinto D, Gobbetti M. 2012. Selected lactic acid bacteria synthesize antioxidant peptides during sourdough fermentation of cereal flours. Appl Environ Microbiol 78:1087–1096.

Coda R, Rizzello CG, Curiel JA, Poutanen K, Katina K. 2014a. Effect of bioprocessing and particle size on the nutritional properties of wheat bran fractions. Innov Food Sci Emerg Technol 25:19–27.

Coda R, Kärki I, Nordlund E, Heiniö R-L. 2014b. Influence of particle size on bioprocess induced changes on technological functionality of wheat bran. Food Microbiol 37:69–77.

Corsetti A, Settanni L. 2007. Lactobacilli in sourdough fermentation. Food Res Int 40:539–558.

Coulibaly A, Kouakou B, Chen J. 2011. Phytic acid in cereal grains: structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. Am J Plant Nutr Fertil Technol 1:1–22.

Courtin CM, Delcour JA. 2002. Arabinoxylans and endoxylanases in wheat flour bread-making. J Cereal Sci 35:225–243.

Crowe JH, Carpenter JF, Crowe LM, Anchordoguy TJ. 1990. Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology 27:219–231.

Damodaran S. 2008. Amino acids, peptides and proteins. In: Damodaran S, Parkin Fennema, O. R. KL, Damodaran S, editors. Fennema's Food Chemistry. 4th Ed. Boca Raton (FL): CRC Press. p. 217–330.

Day L. 2013. Proteins from land plants – Potential resources for human nutrition and food security. Trends Food Sci Technol 32:25–42.

De Angelis M, Coda R, Silano M, Minervini F, Rizzello CG, Di Cagno R, Vicentini O, De Vincenzi M, Gobbetti M. 2006. Fermentation by selected sourdough lactic acid bacteria to decrease coeliac intolerance to rye flour. J Cereal Sci 43:301–314.

De Brier N, Gomand S V., Celus I, Courtin CM, Brijs K, Delcour JA. 2015. Extractability and chromatographic characterization of wheat (*Triticum aestivum* L.) bran protein. J Food Sci 80:C967–974.

De Vuyst L, Neysens P. 2005. The sourdough microflora: biodiversity and metabolic interactions. Trends Food Sci Technol 16:43–56.

De Vuyst L, Van Kerrebroeck S, Harth H, Huys G, Daniel H-M, Weckx S. 2014. Microbial ecology of sourdough fermentations: Diverse or uniform? Food Microbiol 37:11–29.

Delcour JA, Hoseney RCM-C (Eds.). 2010. Principles of Cereal Science and Technology. 3th Ed. St. Paul (MN): AACC International, Inc.

Di Lena G, Patroni E, Quaglia GB. 1997. Improving the nutritional value of wheat bran by a white-rot fungus. Int J Food Sci Technol 32:513–519.

Dornez E, Gebruers K, Delcour JA, Courtin CM. 2009. Grain-associated xylanases: occurrence, variability, and implications for cereal processing. Trends Food Sci Technol 20:495–510.

Dornez E, Gebruers K, Wiame S, Delcour JA, Courtin CM. 2006. Insight into the distribution of arabinoxylans, endoxylanases, and endoxylanase inhibitors in industrial wheat roller mill streams. J Agric Food Chem 54:8521–8529.

Dornez E, Joye IJ, Gebruers K, Delcour JA, Courtin CM. 2006. Wheat-kernel-associated endoxylanases consist of a majority of microbial and a minority of wheat endogenous endoxylanases. J Agric Food Chem 54:4028–4034.

Dunaevsky YE, Sarbakanova ST, Belozersky MA. 1989. Wheat seed carboxypeptidase and joint action on gliadin of proteases from dry and germinating seeds. J Exp Bot 40:1323–1329.

El-Adawy TA. 1997. Effect of sesame seed protein supplementation on the nutritional, physical, chemical and sensory properties of wheat flour bread. Food Chem 59:7–14.

Elliott D, J. Orth R, A. Werpy T, Gao J, E. Eakin D, J. Schmidt A, G. Neuenschwander G, Murry J, Flagg A, Lahman L, Mennel D, J. Lin C. 2002. Biorefinery concept development based on wheat flour milling. Fuel Chem Div Prepr 47:341–362.

[EU] European Union. 2006. Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. Official Journal of the European Union 30.12.2006. L 404.

Every D, Simmons L, Al-Hakkak J, Hawkins S, Ross M. 2002. Amylase, falling number, polysaccharide, protein and ash relationships in wheat millstreams. Euphytica 126:135–142.

Fabian C, Ju Y-H. 2011. A review on rice bran protein: its properties and extraction methods. Crit Rev Food Sci Nutr 51:816–827.

[FAO] Food and Agriculture Organization of the United Nations. 2019a. FAO Cereal supply and demand brief [WWW Document]. URL http://www.fao.org/worldfoodsituation/csdb/en/ (assessed 27.2.2019)

[FAO] Food and Agriculture Organization of the United Nations. 2019b. FAOSTAT Database [WWW Document]. URL http://www.fao.org/faostat/en/#data/QC (assessed 1.3.2019)

[FAO] Food and Agriculture Organization of the United Nations. 2002. Food energy – methods of analysis and conversion factors. FAO Food and nutrition paper 77. Rome, Italy.

Fardet A. 2010. New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre? Nutr Res Rev 23:65–134.

Fath A, Bethke P, Lonsdale J, Meza-Romero R, Jones R. 2000. Programmed cell death in cereal aleurone. Plant Mol Biol 44:255–266.

Faulds CB, Zanichelli D, Crepin VF, Connerton IF, Juge N, Bhat MK, Waldron KW. 2003. Specificity of feruloyl esterases for water-extractable and water-unextractable feruloylated polysaccharides: influence of xylanase. J Cereal Sci 38:281–288.

Galleschi L, Felicioli F. 1994. Purification, characterization and activation by anions of an aspartic proteinase isolated from bran of soft wheat. Plant Sci 98:15–24.

Gänzle MG. 2014. Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol 37:2–10.

Gänzle MG, Loponen J, Gobbetti M. 2008. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. Trends Food Sci Technol 19:513–521.

Gao L, Wang A, Li X, Dong K, Wang K, Appels R, Ma W, Yan Y. 2009. Wheat quality related differential expressions of albumins and globulins revealed by two-dimensional difference gel electrophoresis (2-D DIGE). J Proteomics 73:279–296.

Gebruers K, Debyser W, Goesaert H, Proost P, Van Damme J, Delcour JA. 2001. *Triticum aestivum* L. endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities. Biochem J 353:239–244.

Gobbetti M, Gänzle MG. 2013. Handbook on Sourdough Biotechnology. New York (NY): Springer.

Gobbetti M, Smacchi E, Corsetti A. 1996. The proteolytic system of *Lactobacillus sanfrancisco* CB1: purification and characterization of a proteinase, a dipeptidase, and an aminopeptidase. Appl Environ Microbiol 62:3220–3226.

Graf E, Eaton JW. 1990. Antioxidant functions of phytic acid. Free Radic Biol Med 8:61–69.

Greffeuille V, Abecassis J, Lapierre C, Lullien-Pellerin V. 2006. Bran size distribution at milling and mechanical and biochemical characterization of common wheat grain outer layers: a relationship assessment. Cereal Chem J 83:641–646.

Guan X, Yao H. 2008. Optimization of Viscozyme L-assisted extraction of oat bran protein using response surface methodology. Food Chem 106:345–351.

Guan X, Yao H, Chen Z, Shan L, Zhang M. 2007. Some functional properties of oat bran protein concentrate modified by trypsin. Food Chem 101:163–170.

Gys W, Gebruers K, Sørensen J., Courtin C., Delcour J. 2004. Debranning of wheat prior to milling reduces xylanase but not xylanase inhibitor activities in wholemeal and flour. J Cereal Sci 39:363–369.

Hamada JS. 2000. Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. J Food Sci 65:305–310.

Hammes WP, Brandt MJ, Francis KL, Rosenheim J, Seitter MFH, Vogelmann SA. 2005. Microbial ecology of cereal fermentations. Trends Food Sci Technol 16:4–11.

Hanmoungjai P, Pyle D, Niranjan K. 2002. Enzyme-assisted water-extraction of oil and protein from rice bran. J Chem Technol Biotechnol 77:771–776.

Haros M, Rosell CM, Benedito C. 2002. Effect of different carbohydrases on fresh bread texture and bread staling. Eur Food Res Technol 215:425–430.

Harris PJ, Chavan RR, Ferguson LR. 2005. Production and characterisation of two wheat-bran fractions: an aleurone-rich and a pericarp-rich fraction. Mol Nutr Food Res 49:536–545.

Hartikainen K, Poutanen K, Katina K. 2014. Influence of bioprocessed wheat bran on the physical and chemical properties of dough and on wheat bread texture. Cereal Chem 91:115–123.

Heiniö R-L, Nordlund E, Poutanen K, Buchert J. 2012. Use of enzymes to elucidate the factors contributing to bitterness in rye flavour. Food Res Int 45:31–38.

Hemdane S, Jacobs PJ, Dornez E, Verspreet J, Delcour JA, Courtin CM. 2016. Wheat (*Triticum aestivum* L.) bran in bread making: A critical review. Compr Rev Food Sci Food Saf 15:28–42.

Hemery Y, Chaurand M, Holopainen U, Lampi A-M, Lehtinen P, Piironen V, Sadoudi A, Rouau X. 2011. Potential of dry fractionation of wheat bran for the development of food ingredients, part I: Influence of ultra-fine grinding. J Cereal Sci 53:1–8.

Hemery Y, Rouau X, Dragan C, Bilici M, Beleca R, Dascalescu L. 2009a. Electrostatic properties of wheat bran and its constitutive layers: Influence of particle size, composition, and moisture content. J Food Eng 93:114–124.

Hemery Y, Lullien-Pellerin V, Rouau X, Abecassis J, Samson M-F, Åman P, Reding W, Spoerndli C, Barron C. 2009b. Biochemical markers: Efficient tools for the assessment of wheat grain tissue proportions in milling fractions. J Cereal Sci 49:55–64.

Hemery Y, Rouau X, Lullien-Pellerin V, Barron C, Abecassis J. 2007. Dry processes to develop wheat fractions and products with enhanced nutritional quality. J Cereal Sci 46:327–347.

Hettiarachchy NS, Griffin VK, Gnanasambandam R. 1996. Preparation and functional properties of a protein isolate from defatted wheat germ. Cereal Chem 73:364–367.

Hug-Iten S, Escher F, Conde-Petit B. 2003. Staling of bread: Role of amylose and amylopectin and influence of starch-degrading enzymes. Cereal Chem 80:654–661.

Idris WH, Babiker EE, El Tinay AH. 2003. Fractionation, solubility and functional properties of wheat bran proteins as influenced by pH and/or salt concentration. Nahrung / Food 47:425–429.

Jääskeläinen A-S, Holopainen-Mantila U, Tamminen T, Vuorinen T. 2013. Endosperm and aleurone cell structure in barley and wheat as studied by optical and Raman microscopy. J Cereal Sci 57:543–550.

Jerkovic A, Kriegel AM, Bradner JR, Atwell BJ, Roberts TH, Willows RD. 2010. Strategic distribution of protective proteins within bran layers of wheat protects the nutrient-rich endosperm. Plant Physiol 152:1459–1470.

Jiamyangyuen S, Srijesdaruk V, Harper JW. 2005. Extraction of rice bran protein concentrate and its application in bread. J Sci Technol 27:55–64.

Jodayree S, Smith JC, Tsopmo A. 2012. Use of carbohydrase to enhance protein extraction efficiency and antioxidative properties of oat bran protein hydrolysates. Food Res Int 46:69–75.

Juntunen KS, Laaksonen DE, Autio K, Niskanen LK, Holst JJ, Savolainen KE, Liukkonen K-H, Poutanen KS, Mykkänen HM. 2003. Structural differences between rye and wheat breads but not total fiber content may explain the lower postprandial insulin response to rye bread. Am J Clin Nutr 78:957–964.

Kakade ML. 1974. Biochemical basis for the differences in plant protein utilization. J Agric Food Chem 22:550–555.

Kamal-Eldin A, Lærke HN, Knudsen K-EB, Lampi A-M, Piironen V, Adlercreutz H, Katina K, Poutanen K, Man P. 2009. Physical, microscopic and chemical characterisation of industrial rye and wheat brans from the Nordic countries. Food Nutr Res 53.

Katina K, Juvonen R, Laitila A, Flander L, Nordlund E, Kariluoto S, Piironen V, Poutanen K. 2012. Fermented wheat bran as a functional ingredient in baking. Cereal Chem J 89:126–134.

Katina K, Laitila A, Juvonen R, Liukkonen K-H, Kariluoto S, Piironen V, Landberg R, Aman P, Poutanen K. 2007. Bran fermentation as a means to enhance technological properties and bioactivity of rye. Food Microbiol 24:175–186.

Katina K, Heiniö R-L, Autio K, Poutanen K. 2006. Optimization of sourdough process for improved sensory profile and texture of wheat bread. LWT - Food Sci Technol 39:1189–1202.

Katina K, Poutanen K, Autio K. 2004. Influence and interactions of processing conditions and starter culture on formation of acids, volatile compounds, and amino acids in wheat sourdoughs. Cereal Chem 81:598–610.

Kies AK, De Jonge LH, Kemme PA, Jongbloed AW. 2006. Interaction between protein, phytate, and microbial phytase. *In Vitro* Studies. J Agric Food Chem: 54: 1753–1758.

Kinsella JE, Melachouris N. 1976. Functional properties of proteins in foods: A survey. C R C Crit Rev Food Sci Nutr 7:219–280.

Klassen DR, Elmer CM, Nickerson MT. 2011. Associative phase separation involving canola protein isolate with both sulphated and carboxylated polysaccharides. Food Chem 126:1094–1101.

Klepacka J, Fornal L. 2006. Ferulic acid and its position among the phenolic compounds of wheat. Crit Rev Food Sci Nutr 46:639–647.

Klupsaite D, Juodeikiene G, Zadeike D, Bartkiene E, Maknickiene Z, Liutkute G. 2017. The influence of lactic acid fermentation on functional properties of narrow-leaved lupine protein as functional additive for higher value wheat bread. LWT 75:180–186.

Knuckles BE, Kuzmicky DD, Betschart AA. 1985. Effect of phytate and partially hydrolyzed phytate on *in vitro* protein digestibility. J Food Sci 50:1080–1082.

Korakli M, Gänzle MG, Vogel RF. 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. J Appl Microbiol 92:958–965.

Korhonen H, Pihlanto A. 2006. Bioactive peptides: Production and functionality. Int Dairy J 16:945–960.

Kriz AL. 1999. 7S globulins of Cereals. In: Shewry PR, Casey R, editors. Seed Proteins. Dordrecht, The Netherlands: Kluwer Academic Publisher. p. 477–498.

Laca A, Mousia Z, Díaz M, Webb C, Pandiella SS. 2006. Distribution of microbial contamination within cereal grains. J Food Eng 72:332–338.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227.

Landberg R, Kamal-Eldin A, Salmenkallio-Marttila M, Rouau X, Åman P. 2008. Localization of alkylresorcinols in wheat, rye and barley kernels. J Cereal Sci 48:401–406.

Lappi J, Selinheimo E, Schwab U, Katina K, Lehtinen P, Mykkänen H, Kolehmainen M, Poutanen K. 2010. Sourdough fermentation of wholemeal wheat bread increases solubility of arabinoxylan and protein and decreases postprandial glucose and insulin responses. J Cereal Sci 51:152–158.

Laubin B, Lullien-Pellerin V, Nadaud I, Gaillard-Martinie B, Chambon C, Branlard G. 2008. Isolation of the wheat aleurone layer for 2D electrophoresis and proteomics analysis. J Cereal Sci 48:709–714.

Laurikainen T, Härkönen H, Autio K, Poutanen K. 1998. Effects of enzymes in fibre-enriched baking. J Sci Food Agric 76:239–249.

Leenhardt F, Levrat-Verny M-A, Chanliaud E, Rémésy C. 2005. Moderate decrease of pH by sourdough fermentation is sufficient to reduce phytate content of whole wheat flour through endogenous phytase activity. J Agric Food Chem 53:98–102.

Levrat-Verny M-A, Coudray C, Bellanger J, Lopez HW, Demigné C, Rayssiguier Y, Rémésy C. 1999. Wholewheat flour ensures higher mineral absorption and bioavailability than white wheat flour in rats. Br J Nutr 82:17–21.

Li Z, Alli I, Kermasha S. 1993. In-vitro α-amylase inhibitor activity-phytate relationships in proteins from Phaseolus beans. Food Res Int 26:195–201.

Lim PE, Tate ME. 1973. The phytases. II. Properties of phytase fractions F1 and F2 from wheat bran and the myo-inositol phosphates produced by fraction F2. Biochim Biophys Acta - Enzymol 302:316–328.

Lioger D, Leenhardt F, Demigne C, Remesy C. 2007. Sourdough fermentation of wheat fractions rich in fibres before their use in processed food. J Sci Food Agric 87:1368–1373.

Lopez HW, Leenhardt F, Coudray C, Remesy C. 2002. Minerals and phytic acid interactions: is it a real problem for human nutrition? Int J Food Sci Technol 37:727–739.

Lopez HW, Krespine V, Guy C, Messager A, Demigne C, Remesy C. 2001. Prolonged fermentation of whole wheat sourdough reduces phytate level and increases soluble magnesium. J Agric Food Chem 49:2657–2662.

Loponen J, Sontag-Strohm T, Venäläinen J, Salovaara H. 2007. Prolamin hydrolysis in wheat sourdoughs with differing proteolytic activities. J Agric Food Chem 55:978–984.

Loponen J. 2006. Prolamin degradation in sourdoughs (dissertation). EKT series 1372. University of Helsinki, Yliopistopaino, Helsinki Finland. https://helda.helsinki.fi/handle/10138/20833

Loponen J, Mikola M, Katina K, Sontag-Strohm T, Salovaara H. 2004. Degradation of HMW glutenins during wheat sourdough fermentations. Cereal Chem 81:87–93.

Maioli M, Pes GM, Sanna M, Cherchi S, Dettori M, Manca E, Farris GA. 2008. Sourdough-leavened bread improves postprandial glucose and insulin plasma levels in subjects with impaired glucose tolerance. Acta Diabetol 45:91–96.

Majzoobi M, Ghiasi F, Habibi M, Hedayati S, Farahnaky A. 2014. Influence of soy protein isolate on the quality of batter and sponge cake. J Food Process Preserv 38:1164–1170.

Manini F, Brasca M, Plumed-Ferrer C, Morandi S, Erba D, Casiraghi MC. 2014. Study of the chemical changes and evolution of microbiota during sourdoughlike fermentation of wheat bran. Cereal Chem J 91:342–349.

Mariotti F, Tomé D, Mirand PP. 2008. Converting nitrogen into protein—Beyond 6.25 and Jones' Factors. Crit Rev Food Sci Nutr 48:177–184.

Mathewson PR. 2000. Enzymatic activity during bread baking. Cereal Foods World 45:98–101.

Mathewson PR, Seaobourn BW. 1983. A new procedure for specific determination of β-amylase in cereals. J Agric food Chem 31:1322–1326.

Matsubara H, Hagihara B, Nakai M, Komaki T, Yonetani T, Okunuki K. 1958. Crystalline bacterial proteinase: II. General properties of crystalline proteinase of *Bacillus subtilis* N'. J Biochem 45:251–258.

Mauron J. 1990. Influence of processing on protein quality. J Nutr Sci Vitaminol (Tokyo) 36 Suppl 1:S57-69.

McCleary B V. 1986. Enzymatic modification of plant polysaccharides. Int J Biol Macromol 8:349–354.

MeinIschmidt P, Ueberham E, Lehmann J, Schweiggert-Weisz U, Eisner P. 2016a. Immunoreactivity, sensory and physicochemical properties of fermented soy protein isolate. Food Chem 205:229–238.

MeinIschmidt P, Sussmann D, Schweiggert-Weisz U, Eisner P. 2016b. Enzymatic treatment of soy protein isolates: effects on the potential allergenicity, technofunctionality, and sensory properties. Food Sci Nutr 4:11–23.

Meziani S, Nadaud I, Gaillard-Martinie B, Chambon C, Benali M, Branlard G. 2012. Proteomic analysis of the mature kernel aleurone layer in common and durum wheat. J Cereal Sci 55:323–330.

Mikola L. 1986. Acid carboxypeptidases in grains and leaves of wheat, *Triticum aestivum* L. Plant Physiol 81:823–829.

Mills ENC, Wilde PJ, Salt LJ, Skeggs P. 2003. Bubble formation and stabilization in bread dough. Food Bioprod Process 81:189–193.

Moore JC, DeVries JW, Lipp M, Griffiths JC, Abernethy DR. 2010. Total protein methods and their potential utility to reduce the risk of food protein adulteration. Compr Rev Food Sci Food Saf 9:330–357.

Moore J, Cheng Z, Su L, Yu L. 2006. Effects of solid-state enzymatic treatments on the antioxidant properties of wheat bran. J Agric Food Chem 54:9032–9045.

Mubarak AE. 2001. Chemical, nutritional and sensory properties of bread supplemented with lupin seed (*Lupinus albus*) products. Nahrung - Food 45:241–245.

Nakano T, Joh T, Tokumoto E, Hayakawa T. 1999. Purification and characterization of phytase from bran of *Triticum aestivum* L. cv. Nourin #61. Food Sci Technol Res 5:18–23.

[NASS] National Agricultural Statistics Service 2018. Flour Milling Products [WWW Report]. National Agricultural Statistics Service, Agricultural Statistics Board, United States Department of Agriculture. URL https://www.nass.usda.gov/Publications/Todays_Reports/reports/cafl1118.pdf (assessed 27.2.2019)

Nikinmaa M, Alam SA, Raulio M, Katina K, Kajala I, Nordlund E, Sozer N. 2017. Bioprocessing of bran with exopolysaccharide producing microorganisms as a tool to improve expansion and textural properties of extruded cereal foams with high dietary fibre content. LWT 77:170–177.

Noort MWJ, van Haaster D, Hemery Y, Schols HA, Hamer RJ. 2010. The effect of particle size of wheat bran fractions on bread quality – Evidence for fibre–protein interactions. J Cereal Sci 52:59–64.

Nordlund E, Katina K, Aura A-M, Poutanen K. 2013. Changes in bran structure by bioprocessing with enzymes and yeast modifies the *in vitro* digestibility and fermentability of bran protein and dietary fibre complex. J Cereal Sci 58:200–208.

Nout MJR. 1994. Fermented foods and food safety. Food Res Int 27:291–298.

O'Dell BL, Boland AR, Koirtyohann SR. 1972. Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J Agric Food Chem 20:718–721.

Okot-Kotber M, Yong K-J, Bagorogoza K, Liavoga A. 2003. Phytase activity in extracts of flour and bran from wheat cultivars: enhanced extractability with β-glucanase and endo-xylanase. J Cereal Sci 38:307–315.

Panfilov V, Karetkin B, Gordienko M, Shakir I. 2015. Optimization of wheat bran medium processing conditions, assessment of biological value for saccharomyces. CBU Int Conf Proc 3:455–460.

Paraskevopoulou A, Provatidou E, Tsotsiou D, Kiosseoglou V. 2010. Dough rheology and baking performance of wheat flour–lupin protein isolate blends. Food Res Int 43:1009–1016.

Parker ML, Ng A, Waldron KW. 2005. The phenolic acid and polysaccharide composition of cell walls of bran layers of mature wheat (*Triticum aestivum* L. cv. Avalon) grains. J Sci Food Agric 85:2539–2547.

Peña-Bautista RJ, Hernandez-Espinosa N, Jones JM, Guzmán C, Braun HJ. 2017. CIMMYT Series on Carbohydrates, Wheat, grains, and health: Wheat-based foods: their global and regional importance in the food supply, nutrition, and health. Cereal Foods World 62:231–249.

Petersson K, Nordlund E, Tornberg E, Eliasson A-C, Buchert J. 2013. Impact of cell wall-degrading enzymes on water-holding capacity and solubility of dietary fibre in rye and wheat bran. J Sci Food Agric 93:882–889.

Pontonio E, Lorusso A, Gobbetti M, Rizzello CG. 2017. Use of fermented milling by-products as functional ingredient to develop a low-glycaemic index bread. J Cereal Sci 77:235–242.

Porta R, Pandey A, Rosell CM. 2011. Enzymes as additives or processing aids in food biotechnology. Enzyme Res 2010:436859.

Poutanen K, Flander L, Katina K. 2009. Sourdough and cereal fermentation in a nutritional perspective. Food Microbiol 26:693–699.

Prosekov A, Babich O, Kriger O, Ivanova S, Pavsky V, Sukhikh S, Yang Y, Kashirskih E. 2018. Functional properties of the enzyme-modified protein from oat bran. Food Biosci 24:46–49.

Prückler M, Siebenhandl-Ehn S, Apprich S, Höltinger S, Haas C, Schmid E, Kneifel W. 2014. Wheat bran-based biorefinery 1: Composition of wheat bran and strategies of functionalization. LWT - Food Sci Technol 56:211–221.

Ramos OS, Malcata FX. 2011. Food-Grade enzymes. In: Moo-Young M, editor. Comprehensive biotechnology. 2nd Ed. Saint Louis (Mo): Pergamon. p. 555–569.

Ranganathan J, Vennard D, Waite R, Dumas P, Lipinski B, Searchinger T, GLOBAGRI-WRR model authors. 2016. Shifting diets for a sustainable food future, Installment 11 of "Creating a Sustainable Food Future." Working paper. World Resources Institute. URL https://www.wri.org/publication/shifting-diets

Regvar M, Eichert D, Kaulich B, Gianoncelli A, Pongrac P, Vogel-Mikus K, Kreft I. 2011. New insights into globoids of protein storage vacuoles in wheat aleurone using synchrotron soft X-ray microscopy. J Exp Bot 62:3929–3939.

Reisinger M, Tirpanalan Ö, Prückler M, Huber F, Kneifel W, Novalin S. 2013. Wheat bran biorefinery – A detailed investigation on hydrothermal and enzymatic treatment. Bioresour Technol 144:179–185.

Rhodes DI, Stone BA. 2002. Proteins in Walls of Wheat Aleurone Cells. J Cereal Sci 36:83–101.

Ring SG, Selvendran RR. 1980. Isolation and analysis of cell wall material from beeswing wheat bran (*Triticum aestivum*). Phytochemistry 19:1723–1730.

Rizzello C, Coda R, Mazzacane F, Minervini D, Gobbetti M. 2012a. Micronized by-products from debranned durum wheat and sourdough fermentation enhanced the nutritional, textural and sensory features of bread. FRIN 46:304–313.

Rizzello C, Nionelli L, Coda R, Gobbetti M. 2012b. Synthesis of the cancer preventive peptide lunasin by lactic acid bacteria during sourdough fermentation. Nutr Cancer 64:111–120.

Rizzello CG, Cassone A, Di Cagno R, Gobbetti M. 2008. Synthesis of angiotensin I-converting enzyme (ACE)-inhibitory peptides and γ-aminobutyric acid (GABA) during sourdough fermentation by selected lactic acid bacteria. J Agric Food Chem 56:6936–6943.

Roberts PJ, Simmonds DH, Wootton M, Wrigley CW. 1985. Extraction of protein and solids from wheat bran. J Sci Food Agric 36:5–10.

Rosa-Sibakov N, Sibakov J, Lahtinen P, Poutanen K. 2015. Wet grinding and microfluidization of wheat bran preparations: Improvement of dispersion stability by structural disintegration. J Cereal Sci 64:1–10.

Rosa NN, Aura A-M, Saulnier L, Holopainen-Mantila U, Poutanen K, Micard V. 2013. Effects of disintegration on *in vitro* fermentation and conversion patterns of wheat aleurone in a metabolical colon model. J Agric Food Chem 61:5805–5816.

Sabillón L, Bianchini A. 2016. From field to table: a review on the microbiological quality and safety of wheat-based products. Cereal Chem J 93:105–115.

Salmenkallio-Marttila M, Katina K, Autio K. 2001. Effects of bran fermentation on quality and microstructure of high-fiber wheat bread. Cereal Chem J 78:429–435.

Santala OK, Nordlund EA, Poutanen KS. 2013. Treatments with xylanase at high (90 %) and low (40 %) water content have different impacts on physicochemical properties of wheat bran. Food Bioprocess Technol 6:3102–3112.

Santala O, Lehtinen P, Nordlund E, Suortti T, Poutanen K. 2011. Impact of water content on the solubilisation of arabinoxylan during xylanase treatment of wheat bran. J Cereal Sci 54:187–194.

Sanz Penella JM, Collar C, Haros M. 2008. Effect of wheat bran and enzyme addition on dough functional performance and phytic acid levels in bread. J Cereal Sci 48:715–721.

Sari YW, Mulder WJ, Sanders JPM, Bruins ME. 2015. Towards plant protein refinery: Review on protein extraction using alkali and potential enzymatic assistance. Biotechnol J 10:1138–1157.

Saulnier L, Guillon F, Chateigner-Boutin A-L. 2012. Cell wall deposition and metabolism in wheat grain. J Cereal Sci 56:91–108.

Selvam R. 2002. Calcium oxalate stone disease: role of lipid peroxidation and antioxidants. Urol Res 30:35–47.

Shchekoldina T, Aider M. 2014. Production of low chlorogenic and caffeic acid containing sunflower meal protein isolate and its use in functional wheat bread making. J Food Sci Technol 51:2331–2343.

Shewry PR, D'Ovidio R, Lafrianda D, Jenkins JA, Mills ENC, Békés F. 2009. Wheat grain proteins. In: Khan K, Shewry PR, editors. Wheat chemistry and technology. 4th Ed. St. Paul (MN): AACC International, Inc. p. 223–298.

Shiferaw B, Smale M, Braun H-J, Duveiller E, Reynolds M, Muricho G. 2013. Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. Food Secur 5:291–317.

Sibakov J, Myllymäki O, Holopainen U, Kaukovirta-Norja A, Hietaniemi V, Pihlava JM, Poutanen K, Lehtinen P. 2011. Lipid removal enhances separation of oat grain cell wall material from starch and protein. J Cereal Sci 54:104–109.

Skylas DJ, Mackintosh JA, Cordwell SJ, Basseal DJ, Walsh BJ, Harry J, Blumenthal C, Copeland L, Wrigley CW, Rathmell W. 2000. Proteome approach to the characterisation of protein composition in the developing and mature wheat-grain endosperm. J Cereal Sci 32:169–188.

Slavin J. 2004. Whole grains and human health. Nutr Res Rev 17:99–110.

Soysal C, Söylemez Z. 2004. Properties of wheat bran polyphenol oxidase. Nahrung - Food 48:5–8.

Sozer N, Nordlund E, Ercili-Cura D, Poutanen K. 2017. Cereal side-streams as alternative protein sources. Cereal Foods World 62:132–137.

Stevenson L, Phillips F, O'Sullivan K, Walton J. 2012. Wheat bran: its composition and benefits to health, a European perspective. Int J Food Sci Nutr 63:1001–1013.

Stone BA. 2006. Cell walls of cereal grains. Cereal Foods World 51:62–65.

Tang S, Hettiarachchy NS, Eswaranandam S, Crandall P. 2003. Protein extraction from heat-stabilized defatted rice bran: II. The role of amylase, Celluclast, and Viscozyme. J Food Sci 68:471–475.

Thiele C, Gänzle MG, Vogel RF. 2002. Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. Cereal Chem 79:45–51.

Thiele C, Grassl S, Gänzle M. 2004. Gluten hydrolysis and depolymerization during sourdough fermentation. J Agric Food Chem 52:1307–1314.

Udenigwe CC, Aluko RE. 2012. Food protein-derived bioactive peptides: production, processing, and potential health benefits. J Food Sci 77:R11–24.

Vatandoust A, Ragaee S, Wood PJ, Tosh SM, Seetharaman K. 2012. Detection, localization, and variability of endogenous β-glucanase in wheat kernels. Cereal Chem 89:59–61.

Vermeulen N, Pavlovic M, Ehrmann MA, Gänzle MG, Vogel RF. 2005. Applied and environmental microbiology. Appl Environ Microbiol 42:786–788.

Vioque J, Sánchez-Vioque R, Clemente A, Pedroche J, Millán F. 2000. Partially hydrolyzed rapeseed protein isolates with improved functional properties. J Am Oil Chem Soc 77:447–450.

Wang M, Hettiarachchy NS, Qi M, Burks W, Siebenmorgen T. 1999. Preparation and functional properties of rice bran protein isolate. J Agric Food Chem 47:411–416.

Waszczynskyj N, Rao CS, Da Silva RSF. 1981. Extraction of proteins from wheat bran: application of carbohydrases. Cereal Chem 58:264–266.

Wehrle K, Arendt EK. 1998. Rheological changes in wheat sourdough during controlled and spontaneous fermentation. Cereal Chem J 75:882–886.

Weiss W, Vogelmeier C, Görg A. 1993. Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma. Electrophoresis 14:805–816.

Wikiera A, Mika M, Grabacka M. 2015. Multicatalytic enzyme preparations as effective alternative to acid in pectin extraction. Food Hydrocoll 44:156–161.

Willett W, Rockström J, Loken B, Springmann M, Lang T, Vermeulen S, Garnett T, Tilman D, DeClerck F, Wood A, Jonell M, Clark M, Gordon LJ, Fanzo J, Hawkes C, Zurayk R, Rivera JA, De Vries W, Majele Sibanda L, Afshin A, Chaudhary A, Herrero M, Agustina R, Branca F, Lartey A, Fan S, Crona B, Fox E, Bignet V, Troell M, Lindahl T, Singh S, Cornell SE, Srinath Reddy K, Narain S, Nishtar S, Murray CJL. 2019. Food in the Anthropocene: the EAT–Lancet Commission on healthy diets from sustainable food systems. Lancet 393:447–492.

Xu L, Diosady LL. 2002. Removal of phenolic compounds in the production of high-quality canola protein isolates. Food Res Int 35:23–30.

Yadav RB, Yadav BS, Chaudhary D. 2011. Extraction, characterization and utilization of rice bran protein concentrate for biscuit making. Br Food J 113:1173–1182.

Yin S-W, Tang C-H, Cao J-S, Hu E-K, Wen Q-B, Yang X-Q. 2008. Effects of limited enzymatic hydrolysis with trypsin on the functional properties of hemp (*Cannabis sativa* L.) protein isolate. Food Chem 106:1004–1013.

Zhang D, Moore WR. 1999. Wheat bran particle size effects on bread baking performance and quality. J Sci Food Agric 79:805–809.

Zhao H-M, Guo X-N, Zhu K-X. 2017. Impact of solid state fermentation on nutritional, physical and flavor properties of wheat bran. Food Chem 217:28–36.

Zhu K-X, Sun X-H, Chen Z-C, Peng W, Qian H-F, Zhou H-M. 2010. Comparison of functional properties and secondary structures of defatted wheat germ proteins separated by reverse micelles and alkaline extraction and isoelectric precipitation. Food Chem 123:1163–1169.

Ziobro R, Witczak T, Juszczak L, Korus J. 2013. Supplementation of gluten-free bread with non-gluten proteins. Effect on dough rheological properties and bread characteristic. Food Hydrocoll 32:213–220.

Zotta T, Piraino P, Ricciardi A, McSweeney PLH, Parente E. 2006. Proteolysis in model sourdough fermentations. J Agric Food Chem 5: 2567–2574

Zurbriggen B, Bailey MJ, Penttillä ME, Poutanen K, Linko M. 1990. Pilot scale production of a heterologous Trichoderma reesei cellulase by *Saccharomyces cerevisiae*. J Biochem 13: 267–278